THE OVARIAN IGF SYSTEM IN THE DOMESTIC HEN (*GALLUS DOMESTICUS*)

Rhys Dafydd Roberts

BSc (Edinburgh)

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Agricultural and Food Research Council,

Institute of Animal Physiology and Genetics Research,

Roslin,

Midlothian,

EH25 9PS.

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ABSTRACT

An assessment was made of the role of insulin-like growth factor-I (IGF-I) in the growth of granulosa and thecal cells of the pre-ovulatory follicles in the ovary of the domestic hen (*Gallus domesticus*).

The presence of IGF-I in granulosa and thecal tissues was demonstrated by radioimmunoassay (RIA) of tissue extracts and by immunocytochemical analysis of cultured cells. Both studies showed that immunoreactive IGF-I was present in granulosa and thecal tissues. Reverse-transcription, polymerase chain reaction and Southern blotting analysis showed that IGF-I mRNA was present in total RNA extracted from granulosa and thecal tissue of the four largest follicles in the follicular hierarchy.

Cell culture systems for chicken granulosa and thecal cells were established and used to determine the effects of IGF-I alone, and with gonadotrophins, on DNA synthesis using the incorporation of [^{3}H]-thymidine into the cell as an index. The results of these studies showed that IGF-I stimulated DNA synthesis in both cell types in a dose-dependent manner. FSH and LH were shown to stimulate steroidogenesis, as measured by RIA, in thecal and granulosa cells respectively, but neither gonadotrophin stimulated DNA synthesis in thecal cells. LH, but not FSH, was shown to have a dose-dependent stimulatory effect on DNA synthesis in granulosa cells; when IGF-I and LH treatments were combined their stimulatory effects were synergistic. Serum factors were also shown to act synergistically with IGF-I in this respect. The effects of IGF-I were found to depend on follicle size, as did the effects of LH on granulosa cells. The synergistic actions of IGF-I and LH with respect to DNA synthesis in granulosa cells were independent of follicular size.

The production of IGF binding proteins (IGFBP) by granulosa and thecal cell cultures was demonstrated using Western ligand blotting. A range IGFBPs were shown to be produced by both cell types; when the patterns were compared, both were found to produce four proteins of the same size, but granulosa cells produced an

additional protein not detected in thecal cell conditioned medium. The stimulation of cultures with IGF-I resulted in increased production of IGFBPs by granulosa but not by thecal cells; stimulation with LH had no effect on IGFBPs by granulosa cells.

Receptor binding studies were performed on granulosa and thecal cell cultures, both cell types were shown to posses IGF-I binding sites from which the peptide was displaceable by insulin and the IGF peptides in a potency order of IGF-I > IGF-II >> insulin. This is a well documented characteristic of an IGF type I receptor. Treatment of granulosa cell cultures with LH had no significant effect on the affinity of IGF-I for these binding sites, or on the number of sites.

In conclusion, IGF-I is produced by, and has significant effects on DNA synthesis and binding protein production in the cells of pre-ovulatory ovarian follicles. Further, these cells have IGF-I binding sites. These observations are consistent with an autocrine or paracrine role for IGF-I in the ovary of the domestic hen.

Declaration

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the planning, execution and presentation of this thesis.

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ABBREVIATIONS

BSA	bovine serum albumin
b.p.	base pairs
c	chicken
c.p.m.	counts per minute
cAMP	cyclic adenosine 5'-monophosphate
DARS	donkey anti-rabbit serum
DAB	3-3'-diaminobenzedene
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
d.p.m.	disintegrations per minute
EDTA	diaminoethanetetra-acetic acid
FCS	foetal calf serum
FSH	follicle stimulating hormone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
h	human
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
IGF	insulin-like growth factor
LH	luteinizing hormone
m	messenger
MOPS	3[N-morpholino]-propanesulphonic acid
M-MLV	Moloney murine leukemia virus

NDS	normal donkey serum
NRS	normal rabbit serum
o	ovine
Pd(N)6	random hexanucleotide mixture.
PBS	phosphate-buffered saline
r.p.m.	revolutions per miute
RNA	ribonucleic acid
RBC	red blood cell
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
TE	Tris-EDTA
TEMED	N, N, N', N-Tetramethylethylenediamine
U.V.	ultra violet
v/v	volume by volume
w/v	weight by volume

PREFACE

The process of ovarian growth and development has been extensively studied. The developmental events preceding ovulation have an important bearing on reproductive success and have consequently been the focus of attention for research in reproduction in man and animals. Despite extensive study, the endocrine and cellular interactions involved are still not completely understood. In poultry, the ovary differs from its mammalian counterpart in anatomy, development, control of ovulation and endocrine interactions. An understanding of ovarian development in poultry is of particular significance because of the economic importance of egg production.

Insulin-like growth factor-I (IGF-I) is present in the blood of many animals (Daughaday *et al.*, 1985; Daughaday and Rotwein, 1989) and in many other body fluids and tissues. It has been shown to regulate the development of cells prior to ovulation in several mammalian species.

The importance of IGF-I in the growth of chickens has been suggested by the work of Huybrechts *et al.* (1985) and Goddard *et al.* (1988) who showed that IGF-I in serum increases during chicken growth. The involvement of IGFs in the physiology of avian reproduction has received little attention in comparison with the mammalian work. The aims of this study were to elucidate the expression of IGF-I, its receptors and binding proteins in chicken ovarian cells and to discover the nature of its role in this environment.

CHAPTER 1: INTRODUCTION

1.1 THE ANATOMY AND DEVELOPMENT OF THE AVIAN OVARY.

1.1.1. Anatomy of the ovary and early development of follicles

The domestic hen has a single functional ovary which is located in the anterior of the body cavity, ventral to the aorta, caudal to the vena cava and adjacent to the cranial extremity of the left kidney and the caudal part of the left lung (King and McLelland, 1975; Gilbert, 1979; Bahr and Johnson, 1991).

Early in embryogenesis the distribution of germ cells between the right and left ovary is unequal, with more colonising the left which then becomes larger than the right one (Gilbert, 1979). From hatching to 4 months the left ovary grows slowly and at this stage all its oocytes are microscopic in size. As hens become sexually active the ovary grows enormously from 0.5 to 60 g (Romanoff and Romanoff, 1949; Amin and Gilbert, 1970). The surface of the left ovary of the laying hen is covered with many large follicles (shown in Figure 1.1). The avian primary oocyte is the largest single cell in the animal kingdom (final weight of 20 g in domestic hen) and the avian ovarian follicle is one of the most rapidly growing structures found in higher vertebrates (King and McLelland, 1975).

At 8 days of embryonic age, the primordial germ cells begin rapid multiplication, at this stage they are referred to as oogonia (Romanoff, 1960). Once these cells reach the prophase of their first meiotic division they become primary oocytes (Franchi *et al.*, 1962; Baker, 1972), this occurs around the time of hatching. The granulosa cells, which are derived from the embryonic ovarian epithelium, align themselves around oocytes between the 4th and 6th day after hatching (Brambell, 1956; Romanoff, 1960; Franchi *et al.*, 1962; Callebaut, 1976). Other cells, probably derived from mesenchymal elements, later add to this structure giving rise to the theca (Brambell, 1956). Within a few weeks of hatch,recognisable follicles about 1 mm in

Figure 1.1 A photograph of an isolated ovary from a domestic laying hen showing the five largest pre-ovulatory follicles (F1 - F5).

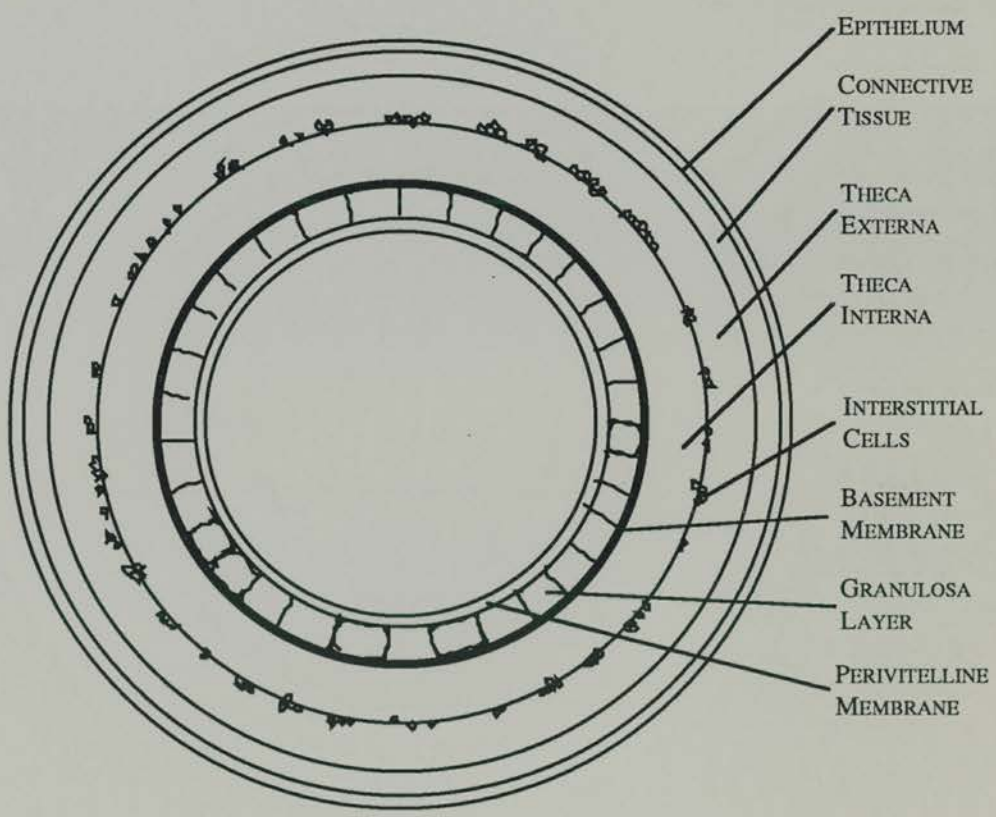

Figure 1.2 A simplified diagram showing the section through a yellow yolky pre-ovulatory follicle in the developing hierarchy. The various cell layers are labelled.

diameter have been produced. Each ovary contains several million follicles (Hutt, 1949).

1.1.2. Follicular structure

Follicles provide physical support for oocytes which grow within them: the structure of the follicular wall is shown in Figure 1.2. The walls of the follicle consist of a single cell layer of granulosa cells immediately surrounding the plasma membrane of the yolky oocyte. The granulosa layer is in turn is surrounded by the basal lamina (which provides physical support and acts as a coarse filter for fluid in the thecal cavity), then by the theca interna layer which consists of large fibroblasts and terminal capillaries. In follicles larger than 5 mm diameter steroidogenic interstitial cells are found in the theca interna, close to the boundary of the two thecal layers (Perry *et al.*, 1978a). The theca interna is surrounded by the theca externa in which the cells are more closely packed and consist of spindle shaped fibroblasts interspersed with considerable quantities of collagen. The thecal layer is surrounded by connective tissue and a superficial epithelium (King and McLelland, 1975). The thecal layers are well vascularised, with capillaries extending as far as the border with the granulosa layer (Rothwell and Solomon, 1977).

1.1.3. Follicular growth

Growth of follicles from 3 to 5 mm takes 3 days, from 5-8 mm, 2 days and from 8 mm to an ovulable size (35-40 mm) 6 days (Gilbert *et al.*, 1985). There are between 4 and 7 yellow-yolky follicles with diameters greater than 8 mm in a developmental hierarchy which are named F1, the next follicle to ovulate, to Fx, the smallest (where x is the total number of follicles in the hierarchy). These follicles are said to be in the "rapid growth" phase

Gilbert *et al.* (1985) analysed the follicular composition of the ovaries of laying hens at the peak of lay. They found that in addition to the largest F1-Fx yellow -yolky follicles, ovaries contained 30-100 small yolky follicles with diameters in the range 1 to

8 mm, 5-25 of these follicles were atretic. In general there were 20 follicles of 1-2 mm diameter and a single follicle of 7-8 mm and between these groups the numbers of follicles in other groups were inversely related to their size. This study revealed that follicular growth is a continuous process which is regulated by differential atresia (see Section 1.1.5. below). Waddington and Walker (1988) found that the follicles which grew larger than 8 mm in diameter were most likely to be located in the anterior portion of the ovary.

As the follicle grows from 1 mm to maturity there is an increase in the oocyte surface area of about 1000 fold; during the final rapid growth phase (9 mm to 35 mm diameter) there is a 15-fold increase (Gilbert *et al.*, 1980). The granulosa layer accommodates this by increasing its own surface area in two ways: an increase in granulosa cell number of approximately 5-fold and an increase in the surface area of each cell by approx 3-fold. Within follicles of 1 mm diameter the granulosa cells are columnar in shape and have the appearance of a pseudostratified epithelium (Bellairs, 1965). As follicles increase in size the granulosa cells change shape to a more flattened pavement type with a larger surface area (Gilbert *et al.*, 1980). During the rapid growth phase, adhesion between granulosa cells is reduced, facilitating the transport of yolk precursors from blood vessels in the theca into the yolk. These changes in the shape of granulosa cells enable them to maintain a uniform covering of the mature oocyte (Perry *et al.*, 1978b).

1.1.4. Yolk deposition

Yolk is formed in the liver under the influence of oestrogen and transported to the ovary as lipoproteins in the blood (Redshaw and Follett, 1972). Yolk deposition occurs in three phases. The first phase covers the period when the follicle grows from 2.5 to 4 mm in diameter, during this phase the yolk is predominantly white in colour. The next phase covers growth from 4 to 8 mm diameter, this is a transition where yolk deposited changes from white to yellow. Most yolk accumulates during growth from 8 mm diameter to ovulation, yellow yolk is deposited in this phase (Griffin *et al.*, 1983).

The composition of white yolk is high in protein and that of yellow yolk is high in lipid; yellow yolk is typified by three fractions which contain phosphoproteins, triglyceride-rich lipoproteins and water soluble proteins (Mackenzie and Martin, 1967; Griffin *et al.*, 1983). The developmentally-related differences in yolk composition are reflected in the change in colour of the follicle from white to yellow as the follicle grows to 8 mm diameter.

1.1.5. Atresia

Atresia is a degenerative process which occurs when a follicle stops growing prior to ovulation. Gupta *et al.* (1988) showed that in follicles smaller than 1 mm in diameter atresia is characterised by resorbtion and that in follicles larger than 1 mm atresia is accompanied by bursting of the follicle. In the same study it was shown that in all atretic follicles examined the oocyte nucleus had degenerated, leading them to suggest that the oocyte is the site at which atretic changes first occur.

The high rate of atresia in follicles between 1 and 8 mm in diameter and the virtual absence of atresia in follicles greater than 8 mm diameter (Gilbert *et al.*, 1983; 1985) suggests that this process controls the numbers of follicles in the smaller population thus constraining the numbers of follicles which may develop to a size greater than 8 mm diameter to one per day; in this way differential atresia creates the hierarchy. Recent work by Tilly *et al.* (1991) has shown that DNA fragmentation occurs in the cells of atretic but not healthy pre-ovulatory follicles. This is a feature of apoptosis or programmed cell death, caused by Ca^{2+}/Mg^{2+}-dependent endonuclease (Wyllie, 1980), thus suggesting that atresia is an apoptotic process; however, the factors which trigger this process are not yet known.

1.1.6. Ovulation and oviposition in the hen

The ovulatory cycle of the hen varies in duration between 24 and 40 hours (Etches, 1990). Ovulation is restricted to a portion of the day known as the "open period" which lasts for approximately 8 hours in chickens (Etches and Schoch, 1984).

Mature follicles occurring outside this period must wait for the next one before they can ovulate, this results in gaps in the laying sequence, referred to as pause days. Fraps (1955) proposed an hypothesis suggesting that the "open period" is caused by a circadian rhythm of hypothalamic sensitivity to an ovulatory signal from the ovary. Further work investigating this hypothesis has shown that progesterone produced by the largest follicle during the LH surge initiates a positive feedback response from the hypothalamus to increase the secretion of GnRH which triggers an increase in the secretion of LH from the adeno-hypophysis. This, in turn, augments the production of progesterone from the largest pre-ovulatory follicle which then builds a pre-ovulatory cascade of progesterone and LH release (Furr and Smith, 1975; Wilson and Sharp, 1975; 1976; Etches and Cunningham, 1976; Johnson *et al.*, 1985). During the "open period" the hypothalamus is believed to increase its sensitivity to the positive feedback actions of progesterone. Although oestrogen is a prerequisite for inducing hypothalamic responsiveness to the stimulatory action of progesterone on LHRH release, it does not create the increased responsiveness to progesterone in the "open period".

Reduction division of the primary oocyte to form the secondary oocyte and the first polar body is completed approximately 2 hours before ovulation. The second maturation spindle then starts to form but mitosis is arrested in metaphase, the division being completed after ovulation and penetration by the sperm (Olsen, 1942). The physical aspects of ovulation are the rupture of the follicle wall in the stigma region followed by expulsion of the yellow yolky oocyte. It has been proposed that the stigma region has a lower tensile strength than the surrounding region due to collagen fibres running parallel with the stigma axis rather than being intertwined (Fuji *et al.*, 1979). Proteolytic enzymes and collagenase are involved in the dissociation of collagen fibres at this time (Ogawa and Goto, 1984).

The secondary oocyte enters the infundibulum where sperm penetration occurs 15 minutes later. From there the fertilised ovum passes through the magnum, isthmus, shell gland, where it stays for 18-20 hours as the shell is laid down, and finally the vagina.

Oviposition follows approximately 24-26 hours after ovulation, the next ovulation in a sequence occurs 15-45 minutes after oviposition (Warren and Scott, 1935). Hens lay eggs daily, forming sequences punctuated by a pause days. These sequences may range in length from 2 to 200; as hens age, the sequence lengths decrease (Bahr and Johnson, 1991).

1.2. ENDOCRINOLOGY OF THE AVIAN OVARY

1.2.1. The ovulatory cycle of mammals and birds

The process of ovulation and the associated ovarian steroidogenesis in birds differs in many details from mammals (Gilbert, 1971; Gilbert and Wells, 1984; Bahr and Johnson, 1991). The events of the ovulatory cycle in poultry occur over hours whereas in mammals the oestrous cycle occurs over a more prolonged period. In mammals the cycle is composed of follicular and luteal phases, in poultry there is no luteal phase since the hormonal support provided by a corpus luteum during pregnancy is not required. In hens the follicular cells appear to have different steroidogenic roles compared with mammals.

1.2.2. The effects of gonadotrophins on ovarian function

The effects of gonadotrophins can be divided into those which are steroidogenic and mitogenic in nature. LH causes increased progesterone secretion by granulosa cells and progesterone is itself responsible for inducing a pre-ovulatory LH surge and ovulation in the chicken (Fraps, 1955; Etches and Cunningham, 1976; Johnson and van Tienhoven, 1984; Lang *et al.*, 1984; Robinson *et al.*, 1988). LH also stimulates production of oestradiol and androstenedione in thecal cells (Marrone and Hertelendy, 1985). FSH induces the theca to secrete predominantly androstenedione (Robinson *et al.*, 1988). Oestradiol is also secreted by the theca of domestic hens when stimulated with ovine FSH, calcium has been shown to be an important mediator in this steroidogenic action of FSH (Ognasbesan and Peddie, 1989). Scanes *et al.* (1977)

reported a rise in concentrations of circulating FSH at approximately 14-15 hours prior to ovulation. However, the measurement of chicken FSH is not very reliable therefore the precise role of this gonadotrophin in preovulatory events is not clear.

The presence of FSH receptors and a responsive adenylyl cyclase system in the ovary has led to speculation that it is involved in follicular selection and growth (Calvo and Bahr, 1983; Ritzhaup and Bahr, 1987). More recently, Yoshimura and Tamura (1988, 1991) have shown that both FSH and LH significantly promote the proliferation of chicken granulosa cells *in vitro*, they also showed that dibutyryl cAMP has a similar effect. These studies suggested that the gonadotrophins stimulate proliferation of granulosa cells via cAMP, a recognised gonadotrophin second messenger (Richards, 1980).

1.2.3. Ovarian steroids

In poultry it has been determined that preovulatory peaks of oestrogen, progesterone and LH occur simultaneously approximately 4-6 hr prior to ovulation (Wilson and Sharp, 1973; Johnson and van Tienhoven, 1980a,b).

The majority of oestrogens produced by the ovary of the laying hen are the product of the small follicles, less than 10 mm diameter (Senior and Furr, 1975; Robinson and Etches, 1986), the rest being produced by the larger follicles. One of the major functions of oestrogens is to sensitize the hypothalamic-pituitary axis to the positive feedback effects of progesterone. (Wilson and Sharp, 1976 - see Section 1.1.6.). Other functions include the stimulation of vitellogenin formation in the liver (Redshaw and Follett, 1972), regulation of calcium metabolism (Etches, 1987), and the maintanence of a functional oviduct and secondary sexual characteristics.

Progesterone is produced by granulosa cells in the five largest preovulatory follicles, this production is stimulated by LH *in vitro*. (Etches *et al.*, 1983). It has been shown to have a direct role in ovulation, specifically in follicular wall breakdown (Tanaka *et al.*, 1987). Progesterone is also required for albumin secretion (Bahr and Johnson, 1991).

Androgens are produced by the small ovarian follicles and all but the largest of the large yolky follicles (Bahr *et al.*, 1983; Robinson and Etches, 1986). The main source of these steroids is the thecal layer although the granulosa cells produce small amounts. Androgens have been shown to induce ovulation in chickens at supra-physiological levels (Fraps, 1955a; Croze and Etches, 1980) and passive immunisation with anti-testosterone serum has been shown to block ovulation (Furr and Smith, 1975). The action of androgens in the ovary are possibly due to the structural similarity of testosterone and progesterone.

1.2.4. Steroidogenesis in the developing follicle

As follicles mature in the final growth phase, progesterone production by the granulosa cells increases and production of androgens and estradiol by the thecal cells decreases (Huang *et al.*, 1979; Etches *et al.*, 1981; Bahr *et al.*, 1983; Etches and Duke, 1984). The granulosa cells of small follicles (F5-F3) have more FSH receptors and produce more progesterone in response to FSH than the granulosa cells of the larger follicles (F1-F2) (Hammond *et al.*, 1981; Calvo & Bahr, 1983; Ritzhaup & Bahr, 1987). In contrast the granulosa cells of the larger follicles are primarily regulated by LH (Calvo *et al.*, 1981; Hammond *et al.*, 1981) Therefore as follicles mature, they progress from an FSH to an LH dominated phase. This alteration in the responsiveness of granulosa cells to FSH and LH is similar to that observed in mammals. Recent work shows that the number of LH receptors in thecal layers drop suddenly on the day of ovulation in japanese quail (Kikuchi and Ishii, 1992); this may account for the drop in production of androgens and oestragen by the theca prior to ovulation (Marrone and Hertelendy, 1985). Following ovulation a post-ovulatory follicle is formed that contains functional granulosa cells, which secrete progesterone; however, the cells of this follicle do not luteinize.

1.2.5. Ovarian cell-cell interactions

An alternative to the two-cell model established for steroid production within the mammalian ovarian follicle (Armstrong and Dorrington, 1977) in which thecal cells produce androgen substrates for granulosa cell synthesis and secretion of oestradiol, has been proposed for steroid production in the chicken ovary (Huang *et al.*, 1979). In the avian two-cell model, granulosa cells are the source of progesterone (production of which is LH dependent) which is then used by the thecal cells to produce androgens and oestrogens under the influence of FSH. This model has been elaborated into a three-cell model (Porter *et al.*, 1989) which suggests that progesterone is converted to testosterone in the theca interna cells and that the theca externa cells convert androgens (including testosterone) into oestradiol by aromatisation. A recent study of the expression of steroidogenic enzymes in the follicle by Nitta *et al.* (1991) broadly supports this theory and also showed that cells in the theca externa are also capable of androgen synthesis.

Perifusion of granulosa layers taken from the largest follices (F1) at different times before they are due to ovulate with LH results in similar progesterone production. However, the co-incubation of theca and granulosa layers from a relatively immature F1 follicles (8 hours of age) results in a suppression of progesterone secretion, whereas co-incubation of these layers from a more mature follicle (32 hours of age) does not (Johnson *et al.*, 1987). Lee and Bahr (1990) found that progesterone production by granulosa cells is suppressed by testosterone and estradiol which act as competitive inhibitors of the enzymes which catalyze progesterone synthesis (P450 side chain cleavage and 3ß-HSD). These two studies show that thecal and granulosa cells interact in the regulation of follicular steroidogenesis.

1.3. THE INSULIN-LIKE GROWTH FACTORS

1.3.1. The somatomedins

The discovery of insulin-like growth factors has several origins. Daughaday and co-workers observed that in hypophysectomised rats, sulphate uptake into cartilage *in vivo* was lower than that in intact rats; however, this deficiency was restored by the administration of growth hormone (GH) (Murphy *et al.*, 1956). Following this study, it was found that serum from hypophysectomised rats administered with GH restored the sulphate incorporation into cartilage *in vitro* to normal levels; however, treatment of the cartilage with GH *in vitro* did not stimulate sulphate uptake (Salmon and Daughaday, 1957). This led the investigators to consider the possibility that the effects of GH were mediated through an intermediate component of serum for which the term "sulphation factor" was proposed. This group then went on to show that sulphation factor effects included the stimulation of collagen synthesis (Daughaday and Mariz, 1962), DNA synthesis (Daughaday and Reeder, 1966) and RNA synthesis (Salmon and DuVall, 1970).

Sulphation factors were first purified by Hall (1972), who, by using bioassays which measured the incorporation of thymidine into cartilage, the insulin-like effects on epidydimal fat (the oxidation of glucose) and the incorporation of sulphate into cartilage, discovered that thymidine factor and insulin-like activity co-purified with sulphation factor. The implication of these experiments was that all three effects were caused by the same molecule. Neutral and basic forms of the peptide were separated and designated somatomedin A and C respectively (Daughaday, 1983).

1.3.2. Non-suppressible insulin-like activity

The second observation which led to the discovery of IGFs was that glucose uptake by the rat diaphragm or adipose tissue was stimulated by serum as if in the presence of insulin (Martin *et al.*, 1958; Renold *et al.*, 1960); but when anti-insulin serum was added 90% of this activity remained (Froesch *et al.*, 1963). This activity

was then termed "non-suppressible insulin-like activity" (NSILA) and it was measured in a bioassay using rat adipose tissue.

The large scale extraction of soluble NSILA (NSILA-s) from Cohn fraction IV was described by Froesch (1983). The resulting crude preparation was purified by Rinderknecht and Humbel (1978 a; b), who isolated two polypeptides, IGF-I and IGF-II in sufficient quantities to determine their amino acid sequences. They found that both peptides closely resembled insulin.

1.3.3. Multiplication stimulating activity

The third observation which led to the discovery of IGFs derives from studies by cell biologists attempting to purify and characterise serum macromolecules required for the multiplication of cells in culture. Insulin was found to stimulatory mitotic activity in tissue cultures in early experiments by Gey and Thalheimer (1924) leading to Temin's observations that insulin could partially replace the serum requirement for the multiplication of chick embryo fibroblasts. However, concentrations of insulin much higher than those found in serum were required (Temin, 1967). These experiments led Pierson and Temin (1972) to search for the serum factor(s) responsible for these insulin-like effects and resulted in the extraction of a low molecular weight fraction which was described as multiplication stimulating activity (MSA). MSA was capable of stimulating DNA synthesis in chicken embryo fibroblasts and possessed insulin-like activity which was not suppressed by insulin antiserum. Analysis of MSA extracted from BRL-3A (buffalo rat liver cell line) conditioned medium by isoelectric focussing and acrylamide gel electrophoresis revealed a family of polypeptides ranging from 7100 to 16250 Da. Two of the species were 7100 Da; it was proposed that the sequences of these species were contained within the larger forms. Marquardt *et al.* (1981) reported the amino acid sequence of a 7,484 Da species of MSA purified from BRL-3A cell line and found it to have only 5 amino acid differences with human IGF-II. They therefore concluded that MSA is the rat homologue of human IGF-II.

1.4. IGF STRUCTURE

1.4.1. IGF-I peptide structure

The insulin-like growth factors are a family of growth factors so named because of their structural and functional similarity to insulin. IGF-I and IGF-II are single chain polypeptides, the mature peptides consisting of 70 and 67 amino acids respectively. IGF-I, IGF-II and insulin are all synthesized from single-chain precursors. Human IGF-I is formed from pre-pro-IGF-I by proteolytic processing at both ends. Similarly IGF-II is formed from human pre-pro-IGF-II. Pre-pro-insulin is cleaved to form pro-insulin in the endoplasmic reticulum, subsequent conversion to insulin involves removal of the C peptide by proteolysis (Steiner, 1977). The formation of insulin from its precursors differs from that of IGF-I and -II since the C-peptides are not removed from them. The amino acid sequences of both human (h)IGF-I and hIGF-II were first determined and published by Rinderknecht and Humbel (1978 a; b) who deduced structural and evolutionary implications which are summarised in Section 1.4.3. below.

The molecular weights calculated from the amino acid sequences were 7649 Da for IGF-I and 7471 Da for IGF-II. There is considerable sequence homology between IGF-I and IGF-II (62%) and the sequences of both closely resemble insulin, 45% of amino acids in these peptides are located in the same positions as in human insulin and the 3 di-sulphide bonds are in the same place in all 3 peptides. Of the 19 invariate residues in insulins, 17 of them are identical in both hIGF-I and hIGF-II; the regions of homology between insulin and IGF-I were identified as the A and B chains of the molecules. Three dimensional models of these molecules have been constructed which indicate that they differ in their antigenic domains (Blundell *et al.*, 1978; Blundell *et al.*, 1983), the probable reason for the lack of binding of IGF-I to anti-insulin antibodies and *vice-versa*.

1.4.2. Comparisons of IGFs from different species.

Since the initial sequencing of the human peptide, the amino acid sequences of IGF-I and IGF-II have been elucidated for a number of organisms including the rat, cow, pig, sheep and chicken (Marquardt *et al.*, 1981; Honegger and Humbel, 1986; Shimatsu and Rotwein, 1987; Francis *et al.*, 1988; 1989a; 1989b; Dawe *et al.* 1988; Armstrong *et al.*, 1990; Ballard *et al.*, 1990) Bovine and porcine IGF-I have identical sequences to that of the human peptide, in the case of sheep there is a single difference. In rats and mice IGF-I differs from human IGF-I in three and four amino acid positions respectively (Bell *et al,* 1986; Shimatsu and Rotwein, 1987). Chickens IGF-I differs from human IGF-I in eight amino acid positions (Ballard *et al.*, 1990). These small inter-species variations in the amino acid sequences of IGFs have led to the suggestion that the peptide plays a fundamentally important biological role (Daughaday and Rotwein, 1989).

1.4.3. Chicken IGF peptides

Dawe *et al.* (1988) purified IGF-I and IGF-II from chicken serum and partially analysed their amino acid sequences. The N-terminal 31 amino acid sequence showed a single substitution at positions 26 with respect to the human peptide. Further analysis of purified IGF-I by Ballard *et al.* (1990) revealed that there were 8 amino acid differences between chicken and human IGF-I at positions 26, 38, 39, 40, 41, 50, 64 and 6. Dawe *et al.* (1988) also partially sequenced two IGF-II variants, one of which had an identical N-terminal hexapeptide to mammalian IGF-II, the N-terminal hexapeptide of the other was considerably different. This was thought to be a result of alternative mRNA splicing. Further analysis of purified cIGF-II by Kallincos *et al.* (1990) found only the cIGF-II variant with the different hexapeptide. They showed that there were marked differences between the amino acid sequences of chicken and mammalian IGF-II especially near the N-terminus, as previously described, and between residues 32 and 40.

1.4.4. Evolution of the IGFs

Rinderknecht and Humbel (1978a;b) found that there were fewer differences between the insulins of major vertebrate classes (birds, reptiles, fish, mammals) than between hIGF-I and these insulins; they also found that the degree of identity between human and rattlesnake insulin and that of the corresponding A and B domains of IGF-I and -II is the same (76%). This led them to suggest that the gene encoding the common ancestor of pro-insulin and IGF-I may have been duplicated before the appearance of vertebrates (600 million years ago).

1.4.5. The IGF-I and IGF-II genes

The characterisation of a cDNA encoding IGF-I was first described by Jansen *et al.* (1983), who screened a human adult liver cDNA library with chemically synthesized oligonucleotides. One of the clones obtained contained an open reading frame of 470 nucleotides, 210 nucleotides of which corresponded to the complete amino acid sequence of IGF-I. The cDNA contained flanking nucleotide sequences corresponding to amino acid residues at both the amino-terminal and carboxy-terminal peptide ends. This suggested that the hIGF-I peptide is synthesized as a precursor (130 amino acids in length) which then undergoes proteolytic processing to form the mature IGF-I peptide, similar to the situation with proinsulin and insulin.

Rotwein (1986) isolated another IGF-I cDNA (IGF-IB) from an adult human liver cDNA library encoding a different C-terminal peptide from that isolated by Jansen *et al.* (1983) (IGF-IA). Studies have shown that the human IGF-I gene consists of at least five exons of which exons 1, 2, 3 and 5 make up IGF-IA cDNA and exons 1, 2, 3 and 4 make up IGF-IB cDNA. These arise by alternative splicing (Rotwein *et al.*, 1986).

IGF-II cDNA clones were first isolated from human liver (Bell et al., 1984) and rat liver (Dull et al., 1984) cDNA libraries. Analysis of these clones led to the prediction that the gene encodes a 180 amino acid precursor molecule (preproIGF-II)

within which the mature IGF-II amino acid sequence (67 amino acids) was located 25 residues from the NH_2-terminal of the larger molecule.

Kajimoto and Rotwein (1990) have cloned and sequenced IGF-I cDNA from Xenopus laevis for which the predicted primary translation product is 153 amino acids. This consists of the sequence of the mature protein flanked by an NH_2-terminal sequence of 48 amino acids and a COOH-terminal sequence of 35 amino acids and indicates that the IGF-I protein precursor has been conserved during vertebrate evolution.

Since the original cloning of IGF-I and IGF-II gene sequences described above, the IGF-I and IGF-II genes have been cloned in a number of species including mouse (Stempien *et al.*, 1986), chicken (Kajimoto and Rotwein, 1989; Fawcett and Bulfield, 1990; Taylor *et al.*, 1991), sheep (Dickson *et al.*, 1991), pig (Tavakkol *et al.*, 1988) and Xenopus (Kajimoto and Rotwein, 1990).

1.4.6. The chicken IGF-I gene

Chicken IGF-I cDNA clones have been sequenced independently by two groups. Kajimoto and Rotwein (1989) isolated and sequenced a chicken IGF-I cDNA 814 nucleotides in length. This sequence led them to suggest that pre-pro chicken IGF-1 resembles mammalian IGF-1A precursors in both length and sequence. The sequence of the mature cIGF-I predicted from the cDNA clones was 70 amino acids in length, 8 of which were different from hIGF-I, agreeing with the sequence published by Ballard *et al.* (1990) described above. In the COOH-terminal E domain, 30 of the 35 residues are identical between chicken and human, the 48 amino acids at the NH_2-terminal showed less homology (77%) with the human counterpart. Further work led this group to suggest that the gene is composed of 4 exons which are transcribed and processed into mRNAs of sizes 1.9 and 2.6 kb (Kajimoto and Rotwein, 1991).

Fawcett and Bulfield (1990) isolated an 860 b.p. cDNA clone from a chicken liver cDNA library with a sequence analagous to human exons 1,2,3 and 5 (similar to hIGF-IA cDNA) with an additional exon (1a) between exons 1 and 2. In another clone

they found evidence of exons 1 and 2 spliced together without 1a in between, implying that there is alternative splicing of cIGF-I mRNAs. The additional exon 1a was not reported by Kajimoto and Rotwein (1989). Northern blot analysis with a probe constucted from the 860 b.p.cDNA revealed a major transcript of 0.65-0.85kb with other bands of 9.5, 8.4, 4.1, 3.6, 3.1 and 1.65-1.75kb. This pattern is quite different from that obtained by Rotwein's group. The amino acid sequence deduced from the 860 b.p cDNA clone of Fawcett and Bulfield (1990) predicts a 130 amino acid peptide in which cIGF-I is flanked by a leader peptide (25 amino acids) and an E peptide (35 amino acids). The predicted mature cIGF-I sequence (70 amino acids) was identical to that described by Kajimoto and Rotwein (1989).

1.4.7. The chicken IGF-II gene.

Chicken IGF-II cDNA has been cloned recently. Taylor *et al.* (1991) screened a chicken embryo cDNA library with a human IGF-II probe containing the complete coding region for prepro-IGF-II. They isolated a clone with a high degree of homology with the second exon of the IGF-II genes in humans, mice and rats. The predicted amino acid sequence from the clone was the same as one of the partial amino acid sequences obtained by Kallincos *et al.* (1990), the other C-terminal sequence being identical to those in all other species studied so far. When this sequence was used to probe RNA extracted from chick embryos, four transcripts were identified with sizes of 1.4, 2.2, 4.7 and 7 kb, the most abundant was the 4.7 kb form. This range of transcripts is similar to that seen in humans and rats. The existence of multiple transcripts indicates the use of alternative promoters, polyadenylation sites and alternative splicing patterns for these transcripts (Sussenbach, 1989). Interestingly, Taylor's group describe the expression of antisense RNA transcripts from the cIGF-II gene. There has been speculation that the these could regulate the level of protein synthesis.

1.4.8. The IGF-I gene promoter

The IGF-I promoter region is yet to be fully elucidated. However, Dickson *et al.* (1991) found that in the sheep, IGF-I mRNA transcription initiated from a point within exon 1A. Kajimoto and Rotwein (1991) identified several transcription initiation sites at the 5' end of the cIGF-I gene within a 74-nucleotide portion of exon 1 that was found to be highly conserved between chickens and mammals. This putative IGF-I promoter when fused with a reporter (luciferase) gene caused enhanced luciferase gene expression, providing evidence of the function of this sequence (Kajimoto and Rotwein, 1991).

1.5. IGF RECEPTORS

The IGFs and insulin elicit similar biological responses including the stimulation of fibroblast DNA synthesis and adipose cell glucose metabolism. These responses are typically non-additive, suggesting that the effects are mediated through the same receptor (Renold *et al.*, 1960; Froesch *et al.*, 1963; Morell and Froesch, 1973).

When partially purified preparations of IGFs became available they were radiolabelled and used to demonstrate specific receptors in placental and liver membranes and fibroblasts (Marshall *et al.*, 1974; Megyesi *et al.*, 1974; Takano *et al.*, 1975; Zapf *et al.*, 1975). Receptors for IGFs and insulin preferentially bind the homologous ligand (Rechler *et al.*, 1980; 1983). Insulin receptors have a weak affinity for IGFs and IGF receptors to have a weak affinity for insulin. Additionally insulin cross-reacts with IGF receptors in some tissues but not in others. This situation was clarified in studies using purified IGF-I and -II which revealed that these peptides bind to two distinct receptor sites in rat liver cells (Rechler *et al.*, 1980; Nissley *et al.*, 1983). Radiolabelled IGF-I binding is inhibited by IGF-I, IGF-II and insulin in decreasing order of potency. Radiolabelled IGF-II binding to receptors is inhibited by IGF-II and high concentrations of IGF-I but not by insulin. Thus IGF receptors are defined by their relative affinities for the three insulin-like peptides. The two distinct receptor types

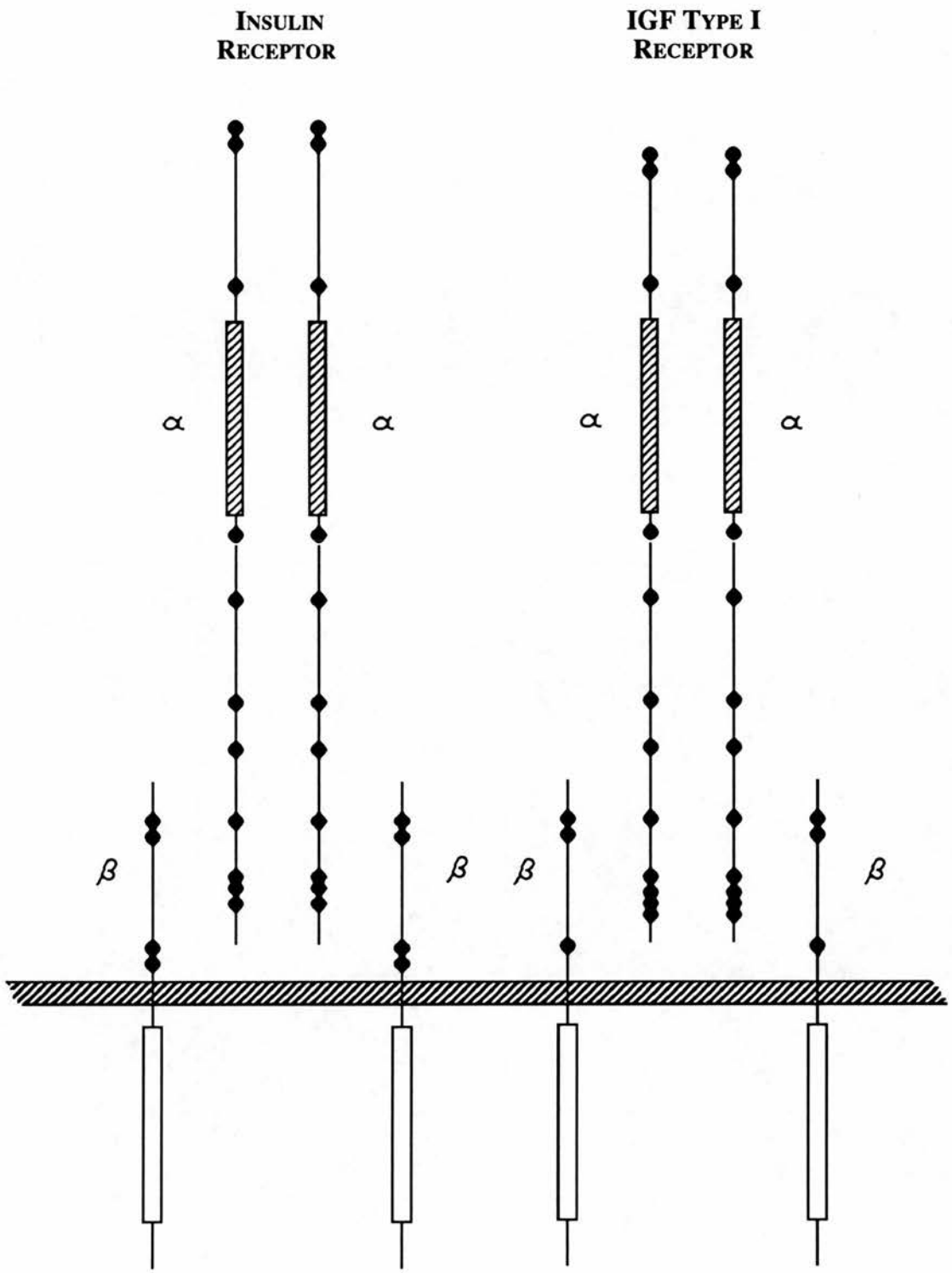

Figure 1.3 Comparison of the structures of human insulin and IGF type-I receptors. The α and β subunits are appropriately marked. Cross-hatched boxes indicate regions of high cysteine residue concentration. Single cysteine residues are shown as black circles. Open boxes indicate tyrosine kinase domains. Ullrich *et al.*,. 1986.

are known as type I and type II receptors, although some authors refer to the type I receptor as an IGF-I receptor and the type II as an IGF-II receptor. Rosenfeld *et al.* (1987) compared cross-reactivity of IGF-I preparations of different purities with the type II receptor and found that the purest IGF-I did not bind to this receptor, leading them to suggest that the previous findings were due to the use of IGF-I preparations which were contaminated with IGF-II.

1.5.1. Receptor structure

1.5.1.1. Type I IGF receptors

The two IGF receptors have been identified by affinity cross-linking studies in which radio-labelled ligands were cross-linked to their respective receptors and then analyzed by SDS PAGE. The type I receptor was found to consist of alpha (α) and beta (β) subunits. The α subunit is 130 kDa in size (Kasuga *et al.*, 1981; Massagué *et al.* 1981; Rechler *et al.* 1983) and the β subunit is 90 kDa (Jacobs *et al.*, 1983; Kull *et al.*, 1983). Massagué and Czech (1982) presented evidence to show that the type I receptor consists of two α subunits and two β subunits connected by disulphide bonds to form a β–α–α–β heterotetromeric complex (Figure 1.3.).

More detailed analysis of structure was made possible when cDNA was cloned from purified receptors. Ullrich *et al.* (1986) deduced the amino acid sequence of the type I receptor from its cDNA and used it to predict that the α subunit is completely extracellular and that the β-subunit has both intracellular and extracellular domains (see Figure 1.3); A similar structure has been described for the insulin receptor (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). In the IGF type I and insulin receptors, the α-subunit contains a cysteine-rich region showing less sequence identity than the surrounding region, suggesting that it is involved in determining the specificity of ligand binding to these receptors. The cytoplasmic domain in type I and insulin receptors contains a region homologous with other tyrosine-specific kinases (Ullrich *et al.*, 1985; Ebina *et*

al., 1985), this is the region most homologous with the insulin receptor and also contains the consensus sequence for an ATP binding site.

Phosphorylation of the β subunit of the type-I receptor is enhanced by IGF-I or by large doses of insulin. Studies with antibodies against receptors, showed that the tyrosyl kinase activity responsible for phosphorylation is intrinsic to the type-I receptor or tightly associated with it (Jacobs *et al.*, 1983b).

1.5.1.2. Type II IGF receptor

In contrast to the type I receptor, the type II receptor is composed of a single polypeptide of Mr 250 kDa (August *et al.*, 1983; Oppenheimer *et al.*, 1983). It has no sequence homology with either the type I or the insulin receptor (Morgan *et al.*, 1987), in fact the sequence is 80% identical with the bovine cation independent-mannose-6-phosphate receptor and studies have shown that these receptors are indeed the same protein (Roth, 1988); 93% of the type II receptor is an extracellular domain and there is no cysteine-rich region.

The type II receptor polypeptide is phosphorylated when rat liver cells are stimulated with IGF-II. Since phosphorylation was not observed in solubilised type II receptors, it seems that the type II receptor does not possess intrinsic protein kinsase activity (Haskell *et al.*, 1984).

1.5.2. The regulation of IGF receptors

Rosenfeld *et al.* (1982b) incubated lymphocytes with IGF-I and noted a reduction of radiolabelled IGF-I binding which, as shown by Scatchard analysis, is caused by a decrease in receptor number. IGF-I, IGF-II and insulin regulate receptor number in proportion to their ability to inhibit rIGF-I binding (i.e in order of potency IGF-I > IGF-II > insulin), suggesting that down-regulation depends on receptor occupancy. IGF-I and insulin have similar effects on type I receptors on human skin fibroblasts (Rosenfeld and Dollar, 1982).

Incubation of mouse muscle cells with IGF-I, IGF-II or insulin does not down-regulate type II receptors (De Vroede *et al.*, 1984); however, incubation of rat adipocytes with insulin causes an increase in IGF-II binding to type II receptors (Schoenle *et al.*, 1976; King *et al.*, 1982; Oppenheimer *et al.*, 1983). Wardzala *et al.* (1984) showed that IGF type II receptors are re-cycled, but when recycling is blocked by KCl, insulin treatment increases the number of type II receptors. The increased binding of IGF-II to adipose cells treated with insulin, therefore, appears to involve a redistribution of type II receptors cycling between an intracellular pool of receptors and the plasma membrane.

Lin *et al.* (1988) found that treatment of rat Leydig cells with hCG, FSH or GH causes an increase in their IGF-I binding capacity which is due to an increase in receptor number rather than affinity. Adashi *et al.* (1988b) also showed that FSH increases IGF-I receptor number on rat granulosa cells, and that the increased IGF-I binding caused by this treatment was enhanced by simultaneous treatment with GH.

1.5.3. IGF receptors in chickens.

Binding sites for IGF peptides have been found in embryonic chicken tissues including fibroblasts and hepatocytes (Rechler *et al.*, 1980; Widmer *et al.*, 1985). Cross-linking studies have shown the presence of an IGF binding site in chicken tissues similar to the type I receptor (Kasuga *et al.*, 1982); however, no evidence has been found for the existence of a type II receptor (Bassas *et al.*, 1988; Canfield and Kornfeld, 1989; Duclos *et al.*, 1991). Duclos and Goddard (1990) examined binding sites for IGF peptides in liver microsomal membranes from young chickens and found that IGF-II has a greater affinity for the receptor than IGF-I but that insulin is also a potent competitor; affinity cross-linking revealed the presence of a 130 kDa polypeptide characteristic of the type I receptor. The evidence suggests that the biological effects of both IGF peptides are mediated through this 'type I' receptor in chickens. Recently, Huybrechts *et al.* (1991) have reported the presence of a type I receptor in chicken

granulosa cells which has equal affinity for IGF-I and -II and a lower affinity for insulin.

1.6. IGF Binding Proteins

1.6.1. IGF-binding protein complexes

The IGF peptides have a molecular weight of 7.5 kDa, but in plasma, serum and other extracellular fluids they are present in both small (30-40 kDa) and large (150 kDa) macromolecular complexes which consist of peptides in association with specific binding proteins (Zapf *et al.*, 1975b; Hintz and Liu, 1977). The structures of these complexes are illustrated in Figure 1.4. The 150 kDa complex consists of three subunits (Baxter and Martin, 1989): an acid labile protein, which appears as a doublet of 84 and 86 kDa on SDS-PAGE (Baxter *et al.*, 1989); an acid stable binding protein and an insulin-like growth factor (-I or -II). In human serum there are two forms of the acid stable protein which have molecular weights of 41.5 and 38.5 kDa respectively (Martin and Baxter, 1986). These represent different glycosylated forms of the protein now known as IGFBP-3. The smaller complexes consist of IGFs with acid stable binding protein subunits which have been named IGFBP-1 to IGFBP-6 (Binoux *et al.*, 1991; Shimasaki and Ling, 1992). The numbers refer to the chronological order in which they were characterised.

As described above, there are two main types of IGF-binding protein complexes. The 150 kDa complex barely crosses the capillary barrier from serum, making its availability to tissues less than the smaller 40 kDa complexes, which cross more easily (Binoux and Hossenlopp, 1988). The half life of IGF is 12-15 hours when associated with the 150 kDa complex, 20-30 minutes with the smaller (40 kDa) molecular weight complexes and 10 to 12 minutes as the free peptide (Guler *et al.*, 1989). Thus, the way in which IGFs are complexed to their binding proteins could determine their availibility to tissues and their stability. The view that binding proteins control the supply of IGF-I to tissues, is supported by the study of Lassare *et al.*

(1991). This showed that the serum of human foetuses and newborn children contains small amounts of IGFBP-3-sized proteins (components of the 150 kDa complex), compared with adult serum; and larger amounts of the smaller IGF binding proteins (components of the 40 kDa complexes). Thus, where the growth rate is high, the type of IGF binding proteins present appear ensure the maximum supply of IGF-I at the tissue level.

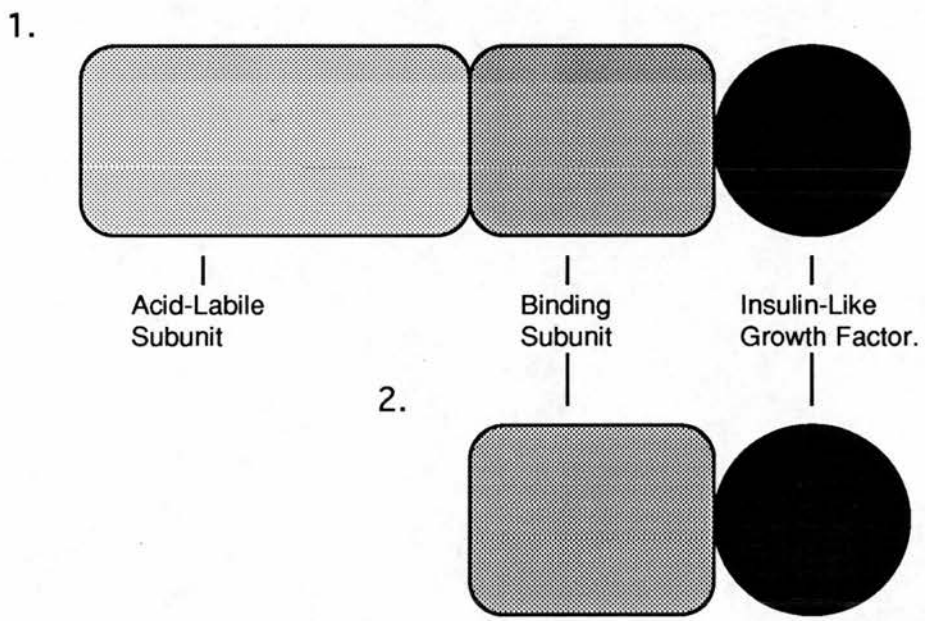

Figure 1.4. The structures of the circulating 150 kDa (1.) and 40 kDa (2.) binding protein comlexes.

1.6.2. Binding protein structure

The binding protein subunits have been the focus of intense investigation in recent years. So far 6 of the smaller binding protein complex subunits have been identified and named in human and rat, and these are described below. Full-length clones encoding the acid-labile subunit have recently been isolated from human liver cDNA libraries, the predicted mature protein sequence is 579 amino acids in length.

These clones have been fuctionally expressed in mammalian tissue culture cells (Leong *et al.*, 1992)

1.6.2.1. IGFBP-1

The first IGF binding protein gene to be cloned and sequenced coded for IGFBP-1 (Brewer *et al.*, 1988; Brinkman *et al.*, 1988; Julkunen *et al.*, 1988; Lee *et al.*, 1988). The cDNA sequence of human IGFBP-1 predicts a mature protein of 234 amino acids and a moecular weight of 25.3 kDa. The apparent molecular mass of the purified protein is 28 kDa (Baxter *et al.*, 1987). The deduced amino acid sequence of rat IGFBP-1 shows 66% homology with the human sequence (Murphy *et al.*, 1990).

1.6.2.2. IGFBP-2

The second IGF binding protein to be cloned and sequenced was named IGFBP-2 (Brown *et al.*, 1989; Margot *et al.*, 1989; Binkert *et al.*, 1989). It has less than 40% homology with hIGFBP-1 and a predicted molecular weight of 31.3 kDa.

1.6.2.3. IGFBP-3

The sequence deduced for the third IGF binding protein, IGFBP-3, predicts a mature protein of molecular mass 28.7 kDa (Wood *et al.*, 1988; Shimasaki *et al.*, 1990a; Albiston and Herrington, 1990). Analysis of the purified protein in human serum shows two bands at 41.5 and 38.5 kDa which are differently glycosylated forms of IGFBP-3 (Binoux *et al.*, 1991). Enzymatic removal of N-linked carbohydrate on IGFBP-3 yields a product of apparent molecular mass about 35 kDa, suggesting that the deglycosylated protein behaves anomalously on SDS-PAGE or that N-glycanase does not fully deglycosylate the protein (Baxter, 1991).

1.6.2.4. IGFBP-4, -5 and -6

The fourth, fifth and sixth binding proteins, IGFBP-4, IGFBP-5 and IGFBP-6 have been cloned and sequenced by Shimasaki and co-workers (Shimasaki *et al.*,

1990a; 1991a;b). The sequence predicted for hIGFBP-4 is 237 amino acids with a molecular weight of 25.7 kDa; rat IGFBP-4 is 4 amino acids smaller, both proteins contain a potential Asn-linked glycosylation site. The sequence predicted for both human and rat IGFBP-5 consists of 252 amino acids with 97% homology, the predicted weights of the mature proteins are 28.4 kDa (rat) and 28.5 kDa (human). The sixth binding protein (IGFBP-6) sequence consists of 216 amino acids for human and 201 for rat with predicted masses of 22.8 kDa and 21.5 kDa, respectively. The sequence for the human protein predicts an Asn-linked glycosylation site.

1.6.2.5. Comparisons of the stuctures of the IGFBPs

The six IGF binding proteins identified so far are distinct members of family of related proteins: features of homology are 18 cysteine residues and conserved amino acid sequence in the amino and carboxyl ends (Rechler and Nissley, 1990; Shimasaki and Ling 1992). IGFBP-6 is slightly anomalous in that it lacks 2 and 4 of the 18 cysteine residues in the rat and human proteins respectively (Shimasaki *et al.*, 1991b). The deduced amino acid sequence also permits insights into the possible functions of these binding proteins. For example, both IGFBP-1 and -2 contain an arg-gly-asp (RGD) sequence (Julkunen *et al.*, 1988; Brown *et al.*, 1989; Binkert *et al.*, 1989) which has been shown to be important in other proteins such as fibronectin and collagen for interaction with integrins, a superfamily of structurally-related receptors, characterised by their abililty to bind ligands containing the RGD tripeptide (Ruoslahti and Pierschbacher, 1987). The presence of RGD in these binding proteins could enhance IGF-I action by aligning the binding protein-IGF complex with the IGF receptor on cells which have integrins on their surfaces. This hypothesis could explain non-enhancing or inhibitory effect of IGFBP3 on IGF action (described in Section 1.6.4.1. below) since its stucture does not contain an RGD sequence.

1.6.3. IGFBP Expression

IGF-binding proteins are expressed in a number of tissues. Immunocytochemical studies in human fetal liver, lung, muscle, kidney, intestine, pancreas, adrenal, skin, bone / growth plate and thymus tissues (Hill *et al.*, 1989b) demonstrated that the cellular distribution of a 26 kDa IGF binding protein and IGF peptides is similar, suggesting that IGFs exist in bound form in, or adjacent to, their sites of action. IGFBP-2 mRNA is found in multiple fetal rat tissues and in adult brain stem, hypothalamus and cerebral cortex (Ooi *et al.*, 1990) and in human cerebro-spinal fluid (Romanus *et al.*, 1989). Endometrial and breast carcinoma cell lines secrete IGFBP-1, -2 and -3 (Camacho-Hubner *et al.*, 1991). Northern analysis of IGFBP-6 mRNA expression shows it to be present in multiple rat tissues indicating a ubiquitous nature (Shimasaki *et al.*, 1991). IGFBP mRNA has been shown to be expressed in rat and human ovaries (Nakatani *et al.*, 1991; Giudice *et al.*, 1991a). Studies of the involvement of IGFBPs in the ovary are discussed in greater depth below.

1.6.4.Regulation of IGFBP expression/production

1.6.4.1. Regulation by GH

The expression of IGF binding proteins is controlled by hormones and growth factors and is dependent on the particular tissue. The dependence of concentrations of IGFBP in serum and isolated rat liver perfusates, on GH, has been demonstrated in the rat (Moses *et al.*, 1976; Schalch *et al.*, 1979; Schwander *et al.*, 1983). *In vitro*, bovine GH caused a 50% increase in IGFBP production by rat hepatocyte monolayers (Scott *et al.*, 1985). The IGFBP produced was not characterised in these studies, however it was postulated to be IGFBP-3 (Baxter and Martin, 1989). Serum concentrations of IGFBP-1 and IGFBP-2 have been found to be inversely related to GH secretory status (Hardouin *et al.*, 1987).

1.6.4.2. Regulation by insulin and the IGFs

The effects of insulin and the insulin-like peptides on the patterns of IGFBP expression in various tissues and cell types has been examined. Hill *et al.* (1989b) showed that the pattern of IGF binding protein expression in human tissues was similar to that of the peptides (see Section 1.6.2. above) suggesting the possibility of mutual regulation. The role of IGFs in the regulation of binding proteins was further elucidated by studies *in vivo* that showed serum concentrations of IGFBP-2 and IGFBP-3 in mice, and IGFBP-3 in rats, are raised by IGF administration (Zapf *et al.*, 1989; Camacho-Hubner *et al.*, 1991a). Studies *in vitro* showed that production of IGFBPs is increased by IGF-I in rabbit articular chondrocytes (30 kDa, 24 kDa and 20 kDa IGFBPs), bovine epithelial cells (IGFBP-3 and IGFBP-2), human fibroblasts (26 kDa IGFBP) and human carcinoma cells (IGFBP-3 and IGFBP-2) (Froger-Gaillard *et al.*, 1989; Hill *et al.*, 1989a; McGrath *et al.*, 1991; Camacho-Hubner *et al.*, 1991b). Production of IGFBPs is inhibited by IGF-I in human decidual cells (IGFBP-1)(Thrailkill *et al.*, 1990). The way in which IGFs regulate their IGFBPs was investigated with human fibroblasts (Hill *et al.*, 1989a; Martin and Baxter, 1990). An IGF analog with reduced receptor affinity stimulated IGFBP-3 secretion from these cells, while an analog with reduced binding protein affinity had minimal effect, implying that the IGF receptor was not important. This was supported by Conover (1991) who investigated the regulation of IGFBP expression by IGF-I using human fibroblasts. Treatment with IGF-I markedly stimulated the appearence of IGFBP-3, a 36 kDa IGFBP and 28-32 kDa IGFBPs and reduced the expression of a 24 kDa IGFBP in conditioned medium. This response was also induced by IGF-I and -II analogues with reduced IGF receptor affinity, but not by an IGF-I analogue with normal receptor affinity and reduced affinity for IGFBP. The response was not induced by high levels of insulin and was not abolished by antibodies to the IGF type-I receptor. The conclusion drawn was that IGF-I regulates IGFBP availibility via a novel receptor-independent mechanism. Cohick and Clemmons (1991) found that the same analogs had opposite effects with bovine epithelial cells, implicating the IGF receptor.

IGFBP concentrations in serum show a diurnal rhythm which is affected by nutritional status; IGFBP-1 concentrations are raised, while those of IGFBP-3 are depressed in patients with diabetes mellitus (Baxter and Martin, 1986; Brismar *et al.*, 1988; Holly *et al.*, 1988; Suikkari *et al.*, 1988). Reviewing the evidence, Lewitt and Baxter (1991) suggested that insulin and hexoses are important independent regulators of plasma IGFBP-1 levels *in vivo*. IGFBP-1 is known to suppress the insulin-like activity of IGFs (Drop *et al.*, 1979; Ritvos *et al.*, 1988). Binding proteins may prevent IGF-I from binding to the insulin receptor and thereby protect against hypoglycaemia caused by the insulin-like effects of IGFs (Daughaday *et al.*, 1980; Zapf *et al.*, 1986; Guler *et al.*, 1988). Insulin has been shown to inhibit IGFBP-1 secretion from rat hepatocytes and human carcinoma cells and to down-regulate both IGFBP-1 and IGFBP-2 mRNA expression in rat liver cells (Boni-Schnetzler *et al.*, 1990; Orolowski *et al.*, 1991; Camacho-Hubner *et al.*, 1991b). However, production of three distinct binding proteins by rabbit chondrocytes was increased by administration of supra-physiological doses of insulin (Froger-Gaillard *et al.*, 1989).

1.6.4.3. Regulation by other factors

Giudice *et al.* (1991b) found that the steroid hormones progesterone and oestradiol and progesterone analogs stimulated the release of IGFBP-2 from human endometrial cells *in vitro* and modulated IGFBP-2 mRNA expression; in the rat ovary FSH has been found to regulate IGF binding protein release from granulosa cells in a biphasic manner (Adashi *et al.*, 1990b; 1991c).

Other factors which have been shown to regulate IGFBP production from cells *in vitro* include TGF-ß (Martin and Baxter, 1991), parathyroid hormone and parathyroid hormone-related peptide (Torring *et al.*, 1991) and forskolin (Cohick and Clemmons, 1991).

1.6.5. Effects of IGFBPs

The IGFBPs were first discovered and characterised by their ability to bind IGF-I and IGF-II and form stable complexes (Cohen and Nissley, 1976; Zapf *et al.*, 1986). There has been a great deal of investigation into the effects of these proteins on the biological activity of IGF-I; some workers suggesting that the formation of complexes with IGFBPs impairs the peptides' ability to interact with their cellular receptors, others suggesting that IGFBPs enhance these interactions.

1.6.5.1. Inhibitory effects of IGFBPs

Initial studies showed that IGFBP purified from human amniotic fluid (32-38 kDa) inhibited the stimulatory effects of the IGFs on fibroblast DNA synthesis (Knauer and Smith, 1980). This effect of binding protein has been confirmed by later work, amongst which was a study involving Des-(1-3)-IGF-I, a peptide identical to IGF-I except for the omission of the amino terminal tripeptide which is required for binding of the peptide to binding protein (Ballard *et al.*, 1987; Szabo *et al.*, 1988). Des-(1-3)-IGF-I has been shown to be more potent than the other IGFs in stimulating DNA synthesis and protein accumulation in some cells; however, in chick embryo fibroblasts, which did not secrete binding proteins, all three peptides were equipotent (Ross *et al.*, 1989). IGFBPs have also been shown to inhibit the effects of IGF-I on glucose oxidation in adipose tissue (Ooi and Herrington, 1986; 1988; Walton *et al.*, 1989), thymidine incorporation in chick embryo fibroblasts (Blat *et al.*, 1989) and FSH-stimulated estradiol synthesis by rat granulosa cells (Shimasaki *et al.*, 1990a).

The inhibitory effects of IGFBPs can be explained by their competition with the receptor for free IGFs. IGFBPs bind to IGFs in a 1:1 molar ratio and compete with the receptor for IGF-I binding in a dose-dependent manner (Ritvos *et al.*, 1988). This observation provides the basis of a mechanism whereby IGFBPs inhibit the actions of IGFs, since this action is mediated through the receptor. The affinity constant of IGFBP-1 for IGF-I is greater than that of the receptor for IGF-I ($Ka = 10^{10}$ and 10^9 M^{-1} respectively, Jones *et al.*, 1991). Although explaining the inhibitory nature of

IGFBPs this hypothesis suggests that IGFs may not bind to the receptor in the presence of binding proteins unless the affinity of IGFBPs for IGF is lowered. Recent studies of the proteolysis and phosphorylation of binding proteins indicate that these processes may regulate the affinity of the binding proteins for IGF-I *in vivo*, thereby releasing the peptide to bind to its receptor. Hossenlopp *et al.* (1990), observed that there is a degradation of IGFBP-3 in the serum and plasma of pregnant women after the second month of gestation and that this could be inhibited by EDTA or aprotinin. This suggests that the degradation is caused by metallo and/or serine proteases.

IGFBP-3 is found in a truncated form *in vivo* (due to proteolysis). Schmid *et al.* (1991) investigated the biological properties of truncated IGFBP-3 in osteoblast cell culture and found that in contrast to full length IGFBP-3 the truncated IGFBP-3 did not inhibit the stimulatory effects of IGF-I on DNA synthesis. These observations provide further evidence that IGFBP-3 is modified by proteolysis *in vivo* and that this could control its affinity for IGF-I.

The effect of phosphorylation of binding proteins was investigated by Jones *et al.* (1991) who showed that phosphorylated IGFBP-1 had an affinity constant 6-fold higher for IGF-I than the same protein following de-phosphorylation. These observations coupled with those showing that IGFBP-1 in hepatoma or decidual cell-conditioned medium is more phosphorylated than that found in fetal serum suggest that the degree of phosphorylation of IGFBP-1 varies *in vivo* and this determines its affinity for IGF-I.

1.6.5.2. Stimulatory effects of IGFBPs

IGFBPs have been shown to increase the binding of IGF-I to its receptor and increase cell proliferation in porcine muscle cells, human and chick fibroblasts (Clemmons *et al.*, 1986; Elgin *et al.*, 1987); and baby hamster kidney cells (Blum *et al.*, 1989). Recombinant hIGFBP-1 enhances IGF-I-induced mitogenesis in human breast carcinoma cells *in vitro* (Camacho-Hubner *et al.*, 1991b). IGFBP-3 influences DNA synthesis in human skin fibroblasts depending on the conditions. Thus, pre-

incubation with IGFBP-3 enhances, while co-incubation inhibits the stimulatory effect of IGF-I on these cells (DeMellow and Baxter, 1988).

Explanations of the stimulatory effects demonstrated by binding proteins are more complicated than those for the inhibitory effects described in Section 1.6.4.1. above. Blum *et al.* (1989) suggested that the slow release of IGFs from binding protein complexes creates a steady state of high IGF receptor occupancy which may elicit a far greater response than that caused by transient levels of IGF. Another explanation is that the RGD sequences in IGFBP-1 and -2 allow the protein to bring IGF closer to the cell surface receptors by binding to integrins on the cell surface (see Section 1.6.1.5. above).

1.6.6. Chicken IGFBP

A protein produced by chick embryo liver cells in tissue culture binds human IGF-I (Hasselbacher *et al.*, 1980) and IGF-I specific binding proteins have been found in chicken serum (Armstrong *et al.*, 1989; Francis *et al.*, 1990) . Armstrong *et al.* (1989) showed that 70% of the IGF-I immunoreactivity in chicken serum is associated with a large protein complex (150 kDa), 20-25% is associated with an intermediate sized complex (45 kDa) and 6% is presumed to be free IGF-I. In the same study, SDS PAGE analysis revealed three IGF-I binding proteins (28.8, 33.1 and 40.7 kDa). Francis*et al.* (1990) injected [^{125}I]-IGF-I into the circulation of chickens and then analysed plasma samples; most of the IGF-I was complexed with binding proteins. By gel permeation chromatography, the circulating binding protein complex in plasma had a molecular mass of 55 kDa. The absence of a 150 kDa complex in the study of Francis *et al.* (1990) conflicts with the findings of Armstrong *et al.* (1989) and may be due to differences in purification which have led to the removal of the complex.

The greater proportion of free IGF-I in chicken serum (6%) in comparison with that in human serum (0.8%) (Daughaday *et al.*, 1982; Armstrong *et al.*, 1989), indicates that birds may be less sensitive to the insulin-like effects of this peptide and

implies that the function of binding proteins in protection from the insulin-like effects of free IGF-I (Zapf *et al.*, 1986) is not as important in birds as in mammals.

1.7. The Mode Of Action Of IGF-I

1.7.1. Endocrine IGF-I actions

In an endocrine mode of action IGFs are synthesized in an organ or tissue and then transported in the circulation to their sites of action. The liver has been regarded as the main source of IGF-I synthesis (e.g. Phillips *et al.*, 1976; Schalch *et al.*, 1979). An endocrine mode of action is supported by evidence that IGF-I is present in the circulation (see Section 1.7.2. below), and that hepatic IGF-I gene expression and peptide synthesis *in vivo* is stimulated by GH (Schwander *et al.*, 1983; Mathews *et al.*, 1986). Further evidence of the endocrine role of the liver is that in humans, it only contains very low levels of IGF-I receptors, probably a pre-requisite to protect it from responding to the locally high concentrations of IGF-I to which it is exposed (Caro *et al.*, 1988).

1.7.2. Paracrine IGF-I actions

In a paracrine mode of action, IGF-I has an effect on tissues or cells which are adjacent to the cells in which it is produced. Evidence of production of IGF-I in tissues other than in the liver has been provided by immunocytochemical and *in situ* hybridisation studies of rat and human tissues respectively (Han *et al.*, 1987; Hansson *et al.*, 1988), these studies show that both IGF-I and its mRNA are produced in most tissue types. Further evidence of a paracrine mode of action for IGF-I comes from the work of Minuto *et al.* (1991) who set up a human tissue culture system in which keratinocytes were grown on fibroblast cell layers. In this system the fibroblasts produced IGF-I but the keratinocytes did not. The medium conditioned by fibroblasts stimulated increased [^{3}H]-thymidine incorporation into keratinocytes, an effect which was inhibited by IGF-I or IGF Type I receptor antibodies. This experiment indicated

that IGF-I produced by fibroblasts has a paracrine effect on keratinocytes *in vitro*. In another study of paracrine interactions, Naville *et al.* (1990) investigated the production of IGF-I by porcine testicular cells under the influence of hCG (amongst other factors). They discovered that hCG stimulated cultures of Leydig and Sertoli cells to produce IGF-I: however, when the two cell types were cultured together hCG stimulated a greater IGF-I production than might be expected from the combined amount produced by separate cultures. These results suggest that there is paracrine regulation of IGF-I production in porcine testes.

1.7.3. Autocrine IGF-I actions

In an autocrine mode of action, IGF-I is synthesized by cells on which it also has an effect. Autocrine mechanisms have been proposed to be responsible for the growth of cancer cells which are believed to produce growth factors for, which they also have receptors, resulting in self-sustained growth stimulation (Sporn and Todaro, 1980). Autocrine actions have been proposed for IGF-I since many cell types which are capable of synthesising IGF-I also possess IGF receptors. IGF-I is known to be mitogenic for many cell types; thus, if it has autocrine actions, normal cells must be able to control them. Cells possibly do this through the production of IGF-I binding proteins. Minuto *et al.* (1991) showed that lung cancer cells secrete an IGF binding protein (CALU-6) which inhibits the mitogenic effects of IGF-I on the lung cancer cells, but not on 3T3 fibroblasts. This indicates an autocrine control mechanism for IGF-I in lung cancer cells.

1.7.4. Relative importance of the endocrine, paracrine and autocrine modes of action

The way in which endocrine, paracrine and autocrine modes of action of IGF-I interact in the whole organism is not fully understood. However, studies of concentrations of serum IGF-I in the human fetus show that it is low compared with the levels in childhood, but there is IGF-I gene expression in many foetal tissues, also GH

receptor numbers rise *post-partum* (Gluckman *et al.*, 1983; Maes *et al.*,1984; Daughaday and Rotwein, 1989). This suggests that the autocrine or paracrine actions predominate over endocrine actions initially, but that the relative importance of the two are developmentally linked. Daughaday and Rotwein (1989) suggested that autocrine/paracrine actions are important in tissues such as lung, kidney and ovarian granulosa cells with high local IGF-I gene expression and IGF-I tissue concentrations.

1.8. IGF-I AND GROWTH

1.8.1 IGF-I and the cell cycle

Serum is required for the growth of cells in culture, and IGF-I is one of the many factors it contains. The role of IGF-I in the growth of cultured cells is to allow progression of the cell cycle from the G0/G1 stage into the S phase (Leof *et al.*, 1982). EGF has a similar function but is only required for the initial phase of the G0 to S stage, while IGF-I is required throughout the stage (Leof *et al.*, 1982; Campisi and Pardee, 1984).

1.8.2. IGFs and growth in chickens

Sulphation factor activity, now known to be IGF-I, was demonstrated in chick embryo and chicken serum by Shapiro and Pimstone, 1977 and Gaspard *et al.*, 1981. The possibility that IGF-I is required for growth in this species was suggested by the work of Huybrechts *et al.* (1985) and Goddard et al. (1988) who showed that total serum immunoreactive IGF-I increases during the rapid phase of chicken growth. Ballard and co-workers (1990) measured the concentrations of IGF-I in the plasma of male broiler chickens and found a two-to-threefold increase in birds from week one to week seven post hatch, the IGF-I peak concentrations being 30-45 ng/ml. When birds were starved for 24 hours the IGF-I levels fell from 40 to 15 ng/ml. No significant difference was found in concentrations of plasma IGF-I between various strains of broilers.

IGF-I serum levels in chick embryos havebeen measured in several studies. De Pablo *et al.* (1991) found that IGF-I concentrations peaked at day 15 (18 ng/ml) and then decreased until hatching but in growth retarded embryos IGF-I remained low (2-4 ng/ml) during the same period. Kikuchi *et al.* (1991) studied embryos from 9 days of age until hatch at 21 days and chicks from hatch up to 7 weeks of age. Their results were similar to those of De Pablo *et al.* (1991) in that they detected a rise in serum IGF-I to a peak of 30-35 ng/ml on day 16, falling back to 10 ng/ml at hatch; they also observed a second peak at six weeks of age (35-40 ng/ml), similar to that observed by Ballard *et al.* (1990). This second rise in IGF-I levels was found to coincide with a rise in GH levels, and in IGF-I mRNA expression in the liver; the pre-hatch peak was not associated with a rise in GH levels, indicating that IGF-I production in chick embryos is independent of GH.

Further evidence of the involvement of IGF-I in the growth of chicken tissues is that IGF-I receptors have been identified in chick embyonic tissues such as brain, heart, muscle, liver and eye (Bassas *et al.*, 1988, 1989). Additionally, Girbau *et al.* (1987) have shown that exogenous insulin and IGF-I stimulate growth-retarded chick embryos between days 2 and 4 of development.

Although the evidence reported above does not show that serum levels of IGF-I are positively correlated with increased growth, it indicates that IGF-I could be important in the growth of chickens at embryonic and post-hatch ages and that GH may be involved in this growth, post-hatch.

1.9. IGFs AND THE OVARY

Recent studies show that insulin and the insulin-like growth factors have an important role in the ovary. Experimentally induced diabetes in female rats is associated with reduced ovarian function (Davoren and Hsueh, 1984), and there is much evidence to suggest that IGF-I (Hammond *et al.*,1982; Hammond *et al.*, 1985; Davoren and Hseuh, 1986) and IGF-II (Voutilainen and Miller, 1987; Ramasharma and Lee, 1987)

are produced by mammalian granulosa cells; subsequently, there has been intense investigation into the particular functions of these peptides in the ovary.

1.9.1. Expression and production of IGFs in the ovary

An immunocytochemical study of rat tissues using antisera specific for IGF-I located the peptide in samples from the ovary, specifically in oocytes, granulosa and the theca interna (Hansson *et al.*, 1988). Studies of mRNA expression have shown that IGF-I mRNA occurs in rat ovarian tissue, specifically in granulosa cells but not in thecal cells. GH increases the level of IGF-I mRNA expression in granulosa cells *in vivo*. A transformed rat granulosa cell line described by Zilberstein *et al.* (1989) has been shown to secrete IGF-I and express IGF-I mRNA. The IGF-I receptor has also been identified in rat granulosa cells (Davoren *et al.*, 1986; Adashi *et al.*, 1988b). IGF-II mRNA expression was not detected in rat ovary (Murphy *et al.*, 1987; Oliver *et al.*, 1989). More recent studies have revealed that IGF-II mRNA is expressed in thecal cells but not in granulosa cells (Hernandez *et al.*, 1990). Human granulosa cells demonstrated the presence of IGF-II mRNA but not IGF-I mRNA (Geisthovel *et al.*, 1989). Thus there are clear differences in the pattern of IGF expression between mammalian species.

Once it had been established that cells of the mammalian ovary produced IGFs, the control of their synthesis by the endocrine system was investigated. As noted above, Hammond and co-workers found that IGF-I is present in follicular fluid and is produced by porcine granulosa cells *in vitro*. This production was found to be stimulated by gonadotrophins and oestrogens (Hammond *et al.*, 1985; Hsu and Hammond, 1987b). Studies by D'Ercole *et al.* (1984) showed that the testicular IGF content in the rat is reduced after hypophysectomy and that this effect was be reversed by administering oGH. Later experiments by Davoren and Hsu (1986) showed that treatment of rats with GH resulted in elevated IGF-I levels in ovarian tissues *in vivo*, implying that GH is also important in the regulation of IGF production in the ovary. Other experiments with porcine granulosa cells *in vitro* showed that treatment with LH,

FSH and oestradiol or GH stimulated IGF-I production as did the growth factors EGF and TGF-α (Hsu and Hammond, 1987a; Mondschein and Hammond, 1988). In the pig, PMSG and hCG treatment resulted in a rise in concentrations of IGF-I in follicular fluid (Hammond *et al.*, 1988). Hsu and Hammond (1987b) found that cAMP analogs had similar effects to gonadotrophin treatments with respect to IGF-I secretion in porcine granulosa cells *in vitro*, implying that this effect of gonadotrophins is mediated by cAMP.

1.9.2. IGF receptors in the ovary

IGF type I receptors have been shown to be present on porcine and rat granulosa cells on rat corpora lutea and on luteinised rat granulosa cells; type II receptors have been shown to be present on rat luteal cells (Baranao and Hammond, 1984; Adashi *et al.*, 1985b; Davoren *et al.*, 1986; Parmer *et al.*, 1991; Talavera and Menon, 1991). Concurrent treatment of FSH with IGF-I, IGF-II or insulin enhances the production of progesterone and oestrogen and the induction of LH/hCG receptors in the order of potency IGF-I > IGF-II > insulin (Davoren *et al.*, 1986). These observations suggest that the biological actions of IGF-I in the rat ovary are mediated preferentially by the type I receptor. The treatment of rats with oestradiol inhibits IGF type I receptor mRNA expression in corpora lutea (Parmer *et al.*, 1991).

The gonadotrophins, FSH and LH, stimulate cAMP production in ovarian cells (Marsh, 1976) and FSH increases IGF-I binding to the type-I receptor on rat granulosa cells *in vitro* and *in vivo* (Adashi *et al.*, 1986b; 1988a; see Section 1.5.2. above). This is due to increased binding capacity rather than increased affinity. These observations are supported by *in situ* hybridisation studies, showing that treatment of rats with PMSG results in an increase in follicular IGF type I receptor mRNA levels (Zhou *et al.*, 1991). A possible conclusion is that FSH up-regulates type I receptors via a mechanism involving cAMP, this is supported by work showing that cAMP-generating agonists have a similar effect to FSH on IGF-I binding (Adashi *et al.*, 1986b).

More recent work by Adashi's group has shown that a GnRH receptor ligand ([D-pGlm^1,D-Phl^2,D-Trp^{3,6}]GnRH) also regulates the binding of IGF-I to the type I receptor in rat granulosa cells (Adashi *et al.*, 1991d), receptor agonists inhibit IGF-I binding by decreasing binding capacity without reducing receptor affinity. The rats used in this study were hypophysectomised to eliminate the involvement of pituitary hormones. GnRH attenuates FSH-stimulated cAMP generation by granulosa cells (Hsueh *et al.*, 1984; Birnbaumer *et al.*, 1985), suggesting that GnRH receptor ligands regulate type-I receptors via cAMP. Antagonists of GnRH receptors have been shown to enhance hCG-stimulated progesterone production by granulosa cells and to increase ovarian weight and protein content (Adashi *et al.*, 1991d).

Thus, there is evidence that the regulation of the type I receptor complement in rat granulosa cells is cAMP dependent and consequently influenced by FSH and GnRH receptor ligands. Adashi *et al.* (1991b) suggested that an intraovarian GnRH system exists; however, identification of the putative endogenous GnRH receptor ligand which may regulate type-I receptors remains elusive.

1.9.3. IGF binding proteins in the ovary

The existence of IGF binding proteins (IGFBPs) in ovarian follicular fluid was first demonstrated by the direct binding of radiolabelled IGF (Hammond, 1981; Hammond *et al.*, 1985). Since then immunoreactive IGFBP-I has been shown to be present in human and porcine ovarian follicular fluid and in human luteinized cells of hyperstimulated preovulatory follicles (Seppala *et al.*, 1984; Seppala and Than, 1987; Mondschein *et al.*, 1991). Human granulosa cells synthesize an IGFBP-1-related species as assessed by incorporation of [^{35}S]-methionine into immunoreactive IGFBP detected by SDS PAGE (Suikkari *et al.*, 1989). In related experiments, acid-chromatographed whole ovarian extracts of murine origin, and media conditioned by porcine granulosa cells were found to contain hormonally-dependent, low molecular weight IGF-I binding activity (Hammond *et al.*, 1985; Davoren and Hsu, 1986). Adashi *et al.* (1990b; 1991c) showed that three IGF binding proteins are released

constitutively by rat granulosa cells *in vitro*, 29, 28 (postulated to be rat IGFBP-2 and IGFBP-1 respectively) and 23 kDa in size. Porcine IGFBP-3 is the most abundant IGFBP in porcine follicular fluid (Mondschein *et al.*, 1991); this protein has been purified and its cDNA cloned (Ui *et al.*, 1989; Shimasaki *et al.*, 1990a). Nakatani *et al.* (1991) used *in situ* hybridisation to study the pattern of IGFBP-1,-2,-3 and -4 mRNA expression in adult rat ovaries. They found that there is tissue-specific synthesis of IGFBP subtypes in specialised ovarian cells; IGFBP-2 is confined to thecal cells, IGFBP-3 is expressed in some corpora lutea and IGFBP-4 is expressed exclusively in the granulosa cells of atretic follicles.

Ovarian IGFBP production is under the control of FSH. Adashi *et al.* (1991c) showed that FSH both stimulates and inhibits the constitutive release of unspecified IGFBPs from rat granulosa cells at different doses (1-3 ng/ml and 10 ng/ml, respectively). The gonadotrophins hCG and FSH have no effect on IGFBP-1 production in human granulosa-luteal cells; however, IGFBP-1 production is stimulated via the protein kinase-C pathway and the cAMP pathway (by PGE_2) (Jalkanen *et al.*, 1989). In the pig, IGFBP-2 expression is correlated with follicular development, IGFBP-2 being more abundant in follicular fluid from small follicles than in fluid from large ones (Mondschein *et al.*, 1991). IGFBP-4 mRNA expression in the granulosa cells of atretic rat follicles is cyclical - varying with the oestrous cycle, indicating that binding proteins are involved with follicular development (Erickson *et al.*, 1992). The most likely mechanism is via control by FSH which has been shown by radioimmunoassay to be present in high concentrations in the follicular fluid of large human pre-ovulatory follicles (McNatty *et al.*, 1975) and to differentially regulate IGFBP expression in rat granulosa cells as described above.

1.9.4. Mechanisms of IGF action in the ovary

Antibodies to insulin have been used to block insulin receptors in porcine granulosa cells *in vitro* (Veldhuis *et al.*, 1984) resulting in the inhibition of the stimulatory effects of insulin on progesterone production. This observation supports

the view that this effect of insulin is mediated by the insulin receptor. However a cross-reaction of the antibody with the IGF type I receptor can not be ruled out, and this idea is supported by the finding that the effects of insulin and IGF-I on progesterone synthesis are non-additive suggesting they act through the same receptor. This view is strengthened by the observation that this IGF-I stimulates progesterone production in granulosa cells at nanomolar concentrations, compatible with its recptor binding affinity as observed in other cell types (Rosenfeld *et al.*, 1984; Adashi *et al.*, 1985b). The observation that IGF-I is much more potent than insulin in augmenting granulosa cell progesterone synthesis (Adashi *et al.*, 1985c) suggests that IGFs act through high affinity recognition sites of their own rather than as insulin surrogates.

Cyclic AMP is generally accepted to be the second messenger for FSH action. Following FSH stimulation of cAMP production, cAMP activates protein kinase A which results in subsequent phosphorylation of key proteins involved in granulosa cell differentiation (Richards *et al.*, 1979). Additional evidence of the involvement of cAMP is that FSH action in granulosa cells is enhanced by co-treatment with phosphodiesterase inhibitors that minimise cAMP breakdown (Welsh *et al.*, 1984). IGF-I alone does not affect cAMP accumulation in conditioned medium from rat granulosa cells, but treatment with both IGF-I and FSH results in increased accumulation compared with that caused by FSH alone, suggesting that the synergy of IGF-I and FSH is mediated in part by cAMP (Adashi *et al.*, 1986b; c).

Recently Dor *et al.* (1991) reported that a woman with IGF-I deficiency had normal ovarian function and suggested that IGF-I is not essential to normal human ovarian development, but that its primary role is as a non-essential modulator of FSH action on granulosa cells. There is also a suggestion from this study that induction of response pathways (e.g. receptor induction) possibly by IGF-I is necessary for normal IGF-I action. However, this is a study based on a single patient which needs to be confirmed before these results can be regarded as significant with respect to IGF-I function in the normal ovary.

1.9.5. The mitogenic effects of IGF-I in the ovary

IGF-I is a potent mitogen for cells derived from primitive germ layers (Clemmons and Van Wyk, 1981) therefore it may be expected to have a role in the multiplication of ovarian somatic cells. However, while the regulation of the steroidogenic functions of ovarian cells by IGFs has been extensively investigated (see Section 1.9.6. below), fewer studies have been undertaken to determine the effects of IGFs on the growth and proliferation of ovarian cells.

Insulin and the insulin-like peptides have been shown to induce dose-dependent mitogenesis in granulosa cells *in vitro* (Veldhuis and Hammond, 1979; Savion *et al.*, 1981; Baranao and Hammond, 1984). May *et al.* (1988) found that IGF-I stimulated thymidine incorporation into DNA of porcine granulosa cells in combination with EGF, but not on its own. A mitogenic effect of insulin on granulosa cells has been described several times (Aidells *et al.*, 1975; May and Schomberg, 1981) but its mitogenic action on granulosa cells *in vitro* is much less than that of IGF-I or IGF-II (Hammond *et al.*, 1983; Baranao and Hammond, 1984; Otani *et al.*, 1985).

1.9.6. Steroidogenic and differentiating effects of IGFs

As the follicle grows and approaches ovulation, the cells in its walls replicate. After ovulation in mammals a corpus luteum forms from the remains of the ovulated follicle. As part of this process granulosa cells become luteinized (see Austin and Short, 1984), that is, they differentiate and become more responsive to LH due to an increase in the number of LH receptors on the cells. The result is that progesterone secretion is enhanced. IGF-I has been shown to promote differentiation in cell types such as chondrocytes (Hill, 1979), myoblasts, (Turo and Florini, 1982) and osteoblasts (Schmidt *et al.*, 1984), suggesting that IGF-I could also have a role in the maturation and development of ovarian cells. The major actions of IGFs which have been studied are progesterone and oestradiol synthesis and the induction of LH receptors. The results of work undertaken to investigate these effects are summarised in the following sections.

1.9.6.1. IGF effects on progesterone synthesis

IGF-I plays a role in the control of progesterone synthesis in the granulosa cells in mammals. It stimulates progesterone secretion directly and also appears to act synergistically with several factors, especially FSH (Adashi *et al.*, 1985b; Russell *et al.* 1984; Baranao and Hammond, 1984). Work done on the effect of IGF-I on granulosa cell progesterone synthesis points to a role in cholesterol side-chain cleavage cytochrome P450 activity. Addition of DNA synthesis inhibitors does not reduce the effect of IGF-I on progesterone production, implying that this effect of IGF-I on steroidogenesis is distinct from its mitogenic effect (Adashi *et al.*, 1985b). FSH-stimulated porcine granulosa cells cultured in serum-free conditions respond to IGF-I, IGF-II and insulin with increased progesterone synthesis, with an overall order of potency of IGF-I > IGF-II > insulin (Channing *et al.*, 1976; May and Schomberg, 1981; Baranao and Hammond, 1984; Adashi *et al.*, 1985b). Progesterone synthesis in porcine thecal cells is also stimulated by insulin treatment (Barbieri *et al.*, 1983).

1.9.6.2. IGF effects on oestrogen synthesis

IGF-I on its own does not affect the development of aromatase activity or oestrogen synthesis by rat granulosa cells, but it synergises with FSH to increase oestrogen synthesis above that produced with FSH alone (Adashi *et al.*, 1985a). Insulin also behaves in this manner (Adashi *et al.*, 1985b). Human granulosa cells have been shown to respond to IGF-I alone with increased oestrogen synthesis (Erickson *et al.*, 1989; Dor *et al.*, 1992). Recent *in vitro* studies of oestradiol production by granulosa cells from a woman with an IGF-I deficiency, indicate that these cells were not as responsive to IGF-I, as cells from normal controls. However, after an extended period in culture the cells did respond well to IGF-I treatment (Dor *et al.*, 1992). Adashi *et al.* (1985a) found that treatment of rat granulosa cells with FSH and IGF-I significantly increased oestrogen accumulation over basal FSH treatment alone. The enhancement of FSH-stimulated oestrogen synthesis by IGF-I is probably acting through aromatase which is stimulated by the growth factor. IGF-II has been shown to

mimic the differentiative effects of IGF-I on cultured rat granulosa cells by stimulating FSH-induced oestrogen production (Davoren *et al.*, 1986) as has insulin at high doses (Hsueh *et al.*, 1989).

1.9.6.3. IGF effects on LH receptor induction

The induction of LH receptors plays a central role in the luteinization of mammalian granulosa cells. FSH is important in this process, since it stimulates increased receptor acquisition (Hsueh *et al.*. 1984, Adashi *et al.*, 1985b). IGF-I synergistically enhances the effect of FSH as do IGF-II and insulin (May and Schomberg, 1981; Adashi *et al.*, 1985b; Davoren *et al.*, 1986). The relative order of potency is IGF-I >IGF-II > insulin. This indicates that insulin and the IGFs are acting via the IGF type I receptor in this case (see Section 1.5 above).

1.9.7. Other effects of IGFs on ovarian cells

Other actions of the insulin-like growth factors on ovarian cells which have been noted in the literature include IGF-I stimulation of proteoglycan synthesis and inhibin production in rat granulosa cells (Adashi *et al.*, 1986a; Zhiwen *et al.*, 1987), oxytocin secretion in bovine granulosa and luteal cells (Schams *et al.*, 1988; McArdle and Holtorf 1989, Holtorf *et al.*, 1989), plasminogen activator activity in chicken granulosa cells (Tilly and Johnson, 1990) and IGF-II stimulation of ornithine decarboxylase activity in porcine granulosa cells (Veldhuis and Hammond, 1979).

Thus, there is evidence that the IGFs have a variety of receptor-mediated effects in ovarian tissues. Combined with the evidence showing that IGFs are also produced here, the conclusion which can be drawn is that they act in an autocrine or paracrine manner in the mammalian ovary.

1.9.8. The synergistic actions of IGF-I and other factors in the ovary

While IGF-I has been shown to exert a direct effect on ovaries, there is evidence that its most potent steroidogenic effects are due to synergistic actions.

In granulosa cells IGF-I has been shown to act synergistically with FSH or PMSG in the in the stimulation of progesterone biosynthesis (Veldhuis and Rodgers, 1987; Veldhuis *et al.*, 1987; Adashi *et al.*, 1988a; Maruo *et al.*, 1988; Mondshein *et al.*, 1989), in the acquisition of aromatase activity and oestrogen accumulation (Adashi *et al.*, 1985a; Maruo *et al.*, 1988; Hutchinson *et al.*, 1988; Erickson *et al.*, 1989), the stimulation of proteoglycan biosynthesis (Adashi *et al.*, 1986a), the stimulation of adenylate cyclase activity (Adashi *et al.*, 1986b), inhibin production (Zhiwen *et al.*, 1987) and LH/hCG receptor binding (Maruo *et al.*, 1988). IGF-I also acts synergistically with FSH, oestradiol, GH, low density lipoprotein or high density lipoproteinin in stimulating progesterone production from porcine granulosa cells (Veldhuis *et al.*, 1987; Mondschein *et al.*, 1989; Veldhuis and Gwynne, 1989). It has recently been shown that progesterone production from chicken granulosa cells stimulated with IGF-I and FSH together was elevated when the cells were pre-incubated with LH (Decuypere *et al.*, 1991). In luteal cells IGF-I acts synergistically with hCG via cholesterol side chain cleavage activity to increase progesterone synthesis (Talavera and Menon, 1991). IGF-II also synergises with FSH in the stimulation of progesterone synthesis in granulosa cells, as does insulin which is much less potent than IGF-I (Baranao and Hammond, 1984; Adashi *et al.*, 1985b). In thecal cells IGF-I acts synergistically with LH or hCG to stimulate progesterone, androstenedione and other androgen production (Barbieri *et al.*, 1983; Hernandez *et al.*, 1988; Caubo *et al.*, 1989).

1.9.9. The interactions of IGF-I and GH in the ovary

The involvement of GH in ovarian function has been indicated in several studies. Suppression of endogenous concentrations of GH in the rat has been shown to affect the onset of puberty, suggesting that this hormone can influence ovarian functions (Ramaley and Phares, 1980; Advis *et al.*, 1981). This view is reinforced by the finding that a reduction of ovarian LH receptor content and hCG-stimulated progesterone production is associated with this reduction of GH concentrations and that

these effects could be reversed by the administration of GH *in vivo* (Bartke *et al.*, 1964; Advis *et al.*, 1981). Additionally, recent work by Gong *et al.* (1991) shows that GH administered to cattle increases the population size of small antral follicles in the ovary.

IGF-I levels have been shown to be influenced by GH; this was the basis of the original somatomedin hypothesis derived from the work of Salmon and Daughaday (1957). Since then, serum concentrations of IGF-I have been shown to be regulated by GH in humans and cattle (Zapf *et al.*, 1981; Grant *et al.*, 1986; Gong *et al.*, 1991). The treatment of GH deficient mice with GH increases IGF-I transcription (Mathews *et al.*, 1986); and treatment of rat hepatocytes with GH *in vitro* causes an increase in IGF-I mRNA expression (Johnson *et al.*, 1989). Thus it is possible that the effects of GH on ovarian functions are mediated via IGF-I. This has been emphasised by experiments demonstrating that GH increases rat ovarian levels of IGF-I *in vivo* (Davoren and Hseuh, 1986) and IGF-I production by porcine granulosa cells *in vitro* (Hsu and Hammond, 1987a). GH has also been shown to enhance FSH-induced differentiation of rat and porcine granulosa cells *in vitro*, as measured by changes in progesterone production and LH receptor formation (Jia *et al.*, 1986; Hsu and Hammond, 1987b), an effect which has been well established for IGF-I (Adashi *et al.*, 1985b; see Sections 1.9.6.1 and 1.9.6.3 above).

A recent study by Mason *et al.* (1990) has shown that GH can have direct steroidogenic effects in human granulosa cells *in vitro* independent of FSH, IGF-I production was undetectable in this study: however, in previous studies gene expression of IGF-I has not been demonstrated in these cells (see Section 1.9.1. above), making them unique among the granulosa cells of the species studied to date. It is possible that GH may act both independently and with IGF-I in the ovary, via separate pathways.

1.10. Research Objectives

The literature reviewed in this introduction shows that IGF-I is widely expressed and is important for the growth of many cell types. It also shows that IGF-I

is involved in mammalian ovarian cell function. Structurally, the mammalian ovary is very different from the avian ovary and the role which IGF-I plays in the regulation of avian ovarian function has not been established. Therefore, it is not known how differences in the morphology of mammalian and avian ovaries may be reflected in the functions of IGF-I in this organ.

The growth of cells is a fundamentally important aspect of the developmental processes that precede ovulation in avian ovarian follicles. In the domestic laying hen, follicles are arranged in a developmental hierarchy in the final 5-7 days before they ovulate; providing an easily visible *in vivo* model of development that can be used to examine the characteristics of growing follicles. Studies on the rat ovary have provided evidence for local IGF-I synthesis and regulation, suggesting that IGF-I has a paracrine or autocrine role in this organ.

Bearing in mind the work which has been done in mammals, and the resulting hypotheses with respect to the function of ovarian IGF-I, a research programme was initiated to establish the role of IGF-I in the chicken ovary. In designing the programme, advantage was taken of the availability at IAPGR of chicken IGF-I cDNA clones. The research objectives of the study were:-

1. To establish whether the cIGF-I gene is expressed in the ovary of the domestic hen, and if so to identify the cellular compartments in which expression occurs.
2. To determine the mitogenic effects of IGF-I in the different compartments of the avian ovary.
3. To examine how the biological effects of IGF-I are related to development within the yellow yolky follicular hierarchy.
4. To establish whether the pre-ovulatory chicken ovarian follicle contains IGF-I receptors and binding proteins.

CHAPTER 2: MATERIALS AND METHODS

2.1. General Materials

2.1.1. Animals

Animals used in all these experiments were ISA Brown laying hens supplied by ISA Poultry Services Ltd. (Peterborough, U.K.). Birds were fed a starter diet (at 1-8 weeks of age), grower diet (9-18 weeks) and a layer diet (19 weeks onwards) as specified by the suppliers; water was available *ad lib*. Birds were killed by cervical dislocation between 50 and 70 weeks of age. The hens used in any one study were of the same age, i.e. they had been reared from the same batch. The birds were kept caged under a 24 hour artificial lighting pattern (14 hours light and 10 hours dark). Egg laying records were kept for each hen and only hens laying daily were selected for study. Most hens laid between 4 and 6 hours after the beginning of the light period. The time of ovulation in these birds was estimated from the time of oviposition, since ovulation occurs about 30 minutes after oviposition of the oviductal egg (Warren and Scott, 1935). Birds were killed in the morning 4 - 6 hours after lights were switched on, after checking for the presence of an egg in the oviduct (by palpation). In this way hens were selected which were predicted to be due to ovulate within 0 - 2 hours.

2.1.2. Reagents

All reagents were obtained from FSA Laboratory Supplies, Loughborough, U.K. and were of analytical grade unless otherwise stated. Water used was purified by pre-filtration, reverse osmosis and further, complex filtration by the Milli-RO and Milli-Q systems (Millipore Waters, Watford, U.K.). Water was further treated as indicated in the individual experimental protocols.

The peptides used in the experiments described below were human recombinant insulin-like growth factors I and II (Bachem, Saffron Walden, U.K.) and porcine monocomponent insulin (Novo Bioloabs Ltd., Cambridge, U.K.). The oLH used was

NIADDK-oLH-25 and the oFSH was NIADDK-oFSH-17, both were gifts from NIADDK (Bethesda, Maryland., U.S.A.). The chicken LH used was preparation AE1, obtained from Dr. P. J.Sharp (IAPGR, Roslin, Midlothian).

The anti oLH (R288) and anti cLH antisera (3/9) were kindly supplied by Dr. R. Webb and Dr. P. J. Sharp respectively (IAPGR, Roslin, Midlothian), who also supplied binding data used to calculate optimal dilutions of the antisera. All other antisera were obtained from the Scottish Antibody Production Unit (Law Hospital, Carluke, U.K.) unless otherwise stated.

2.2. TISSUE CONCENTRATIONS OF IGF-I

2.2.1. Tissue collection

Birds were killed by cervical dislocation. The liver was dissected immediately, samples were weighed and placed in polyurethane microcentrifuge tubes then snap frozen in liquid nitrogen. The ovary was then dissected and the five largest pre-ovulatory follicles (F1, F2, F3, F4 and F5) were removed. The theca and granulosa layers were separated as described below and frozen in the same way as the liver. These tissues were stored at -80°C prior to further processing.

2.2.2 Follicle dissection

Follicles were removed immediately from the ovary and placed in a glass dissection dish containing Dulbecco's phosphate-buffered saline (D-PBS) solution, made up according to the manufacturer's instructions (Oxoid Co., Basingstoke, U.K.) and then autoclaved for 30 minutes at 115°C. The vascular and connective tissue surrounding the follicles was removed with forceps and an incision was made in the follicle in the stigma region allowing most of the yolk to flow out. The remains of the follicle were transferred to a clean dissecting dish containing fresh D-PBS and the granulosa cell layer carefully separated from the thecal layer with forceps

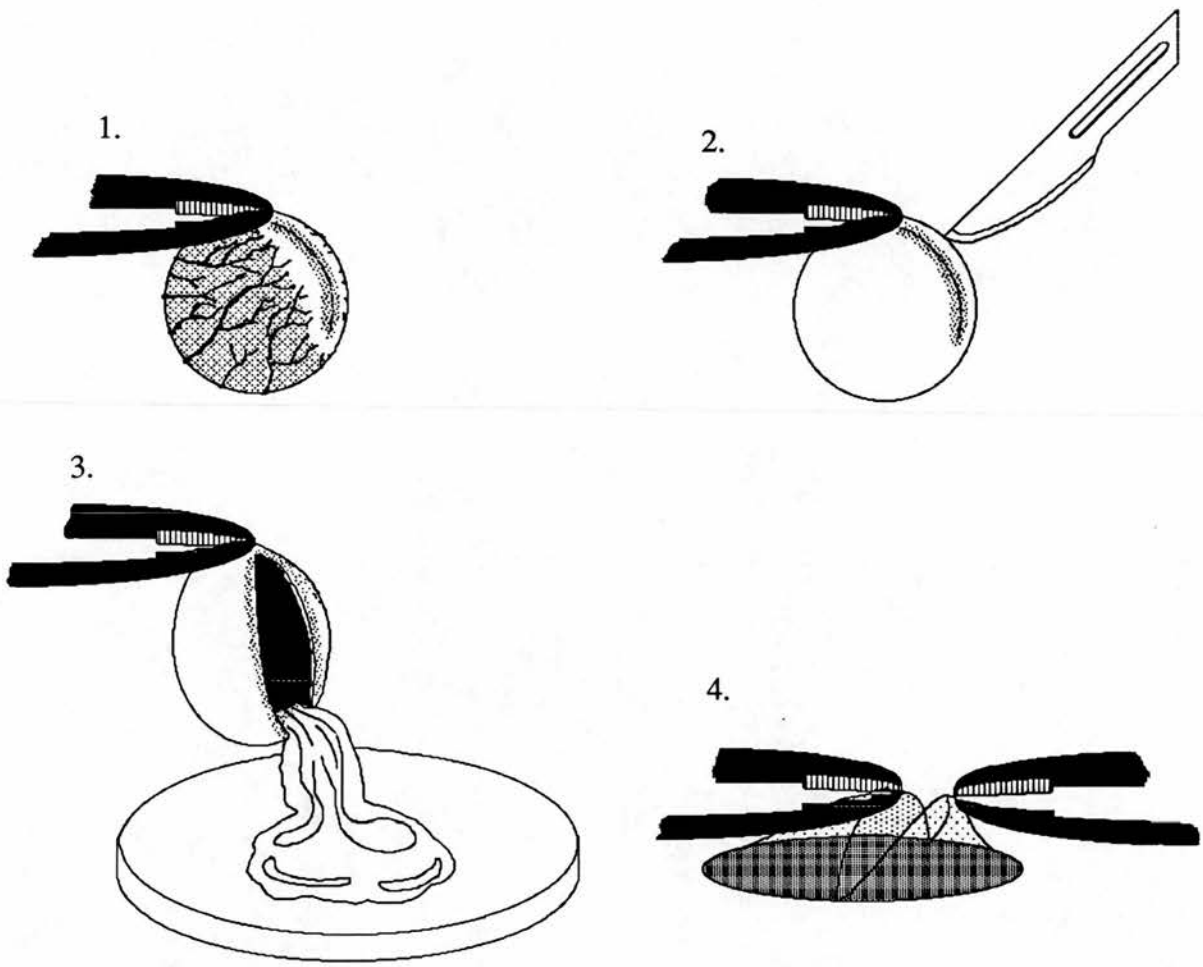

Figure 2.1. Illustration of the method used to separate granulosa and thecal layers. 1. Stroma and exterrnal blood vessels removed. 2. Incision made in stigma. 3. yolk and granulosa removed from theca. 4. Granulosa teased from yolk.

(see Figure 2.1). Both layers were rinsed briefly in D-PBS to remove any adhering yolk and then stored as described in Section 2.2.1. above.

2.2.3 IGF-I Extraction

2.2.3.1 Acid ethanol extraction

This was based on the methods described by by D'Ercole *et al.* (1980) and Enright *et al.* (1989). Tissue samples were homogenised in acetic acid (1 mol/l) using a Polytron homogeniser (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at 1000 g for 15 minutes and the supernatant removed to a clean tube. Ethanol and acetone was then added to the supernatant until the solution had a composition of 60 : 30 : 10, ethanol : acetone : acetic acid. This solution was then vortexed, incubated at room temperature for 30 minutes then centrifuged at 1860 g for 30 minutes at 4°C. The top half of the supernatant was then removed, its pH adjusted to 7.5 with Tris base (4 mol/l) (Sigma Chemical Co., Poole, U.K.) and analysed for IGF-I content by radioimmunoassay (RIA).

2.2.3.2 Acid Chromatography

A chromatography column (30cm x $2cm^2$) was packed with Sephadex G50 superfine (Pharmacia LKB, Milton Keynes, U.K.) which had been pre-swollen in acetic acid (1 mol/l) with protamine sulphate (0.02 % w/v) (Sigma Chemical Co., Poole U.K.) at 90 °C for 60-90 minutes. The column was connected to an HPLC pump and U.V. detector (Millipore Waters, Watford, U.K.) set to detect at a wavelength of 280 nm, fractions were collected with an automatic fraction collector. The column was packed under pressure at a flow rate of at least 1.5 ml/min. in the buffer described above (this buffer was used throughout this procedure).

The column was run at 0.55 ml/min. for several hours and then characterised. The void voume (V_0) was measured by running 0.5 ml of a solution of blue dextran (10 mg/ml)(Pharmacia LKB, Milton Keynes, U.K.) down the column and measuring

the absorbance peak and the volume of buffer eluted, the dextran peak was eluted at a volume of 18 ml, which was then taken as V_0. The separation of proteins by the column was characterised with molecular weight markers; bovine serum albumin (66 kDa)carbonic anhydrase (29 kDa), cytochrome-C (12.4 kDa) and aprotinin (6.5 kDa), all of which were obtained from Sigma Chemical Co. (Poole, U.K.). The two larger proteins were eluted in the void volume, cytochrome-C was eluted at 21.5 ml and aprotinin was eluted at 34.5 ml. The elution of IGF-I from the column and the total volume (Vt) of the column was characterised with [^{125}I]-IGF-I, which eluted between 27 and 36 ml with the peak at 31 ml. The free ^{125}I eluted between 57 and 62 ml, with a peak at 59 ml, therefore the total column volume (Vt) was taken as 59 ml.

The efficiency of the recovery of free IGF-I from the column was calculated by measuring the radioactivity (d.p.m.) in the peak and comparing this with the total d.p.m. of radioactive IGF-I label added. The calculation showed that 70.5% of the total radioactivity was recovered in one column volume and 66% of this was eluted in the "IGF-I" peak.

Tissue samples were homogenised with a Polytron homogeniser in acetic acid (1 mol/l) with Triton X-100 (1% v/v) at 3.5 ml/g of wet tissue. The homogenate was centrifuged at 1000 g for 15 minutes and the supernatant removed in 1 ml aliquots then stored at -20°C.

Samples (500 µl volume) were loaded and the U.V. absorbance was measured. Fractions (1 ml) were collected from the point of sample injection for the total volume of the column. At least 1 column volume of buffer was run through the column in between samples. The fractions were adjusted to pH 7.5 with Tris base (4 mol/l) and assayed for IGF-I by RIA as described below.

2.2.4. IGF-I radioimmunoassay.

The IGF-I assay used was a double antibody RIA using the antiserum R2/2 as previously described by Goddard *et al.* (1988).

Samples and standards (200 µl of each) were dispensed into polystyrene reaction tubes with the anti-IGF-I primary antiserum (R2/2), used at an initial dilution of 1:4000 (100 µl per tube) and then incubated at 4°C for 24 hours. The standards were prepared from recombinant human (h) IGF-I, at concentrations of 10, 60, 100, 200, 400, 600, 800, 1000, 2000 and 4000 pg per tube. Radio-labelled [^{125}I]-IGF-I was added (100 µl per tube) and the tubes vortexed then incubated at 4°C for a further 16 hours. A solution in assay buffer of the donkey-anti-rabbit IgG second antibody, diluted to 1:20, and normal rabbit serum (NRS) carrier, diluted to 1:200, was added to each tube (100 µl) and the tubes incubated for 3 hours at room temperature. The tubes were centrifuged at 1500 g then 1 ml polyethylene glycol (4% w/v) (Sigma Chemical Co., Poole, U.K.) was added prior to re-centrifugation. The supernatant was aspirated and the radioactivity of the pellets measured using a Gammamaster gamma counter (Pharmacia LKB, Milton Keynes, U.K.). The reaction was kept on ice during all additions and in a 4°C cold room during incubations until the addition of the second antibody. The assay buffer was composed of sodium dihydrogen orthophosphate (26 mmol/l), EDTA disodium salt (10 mmol/l), polyoxyethylenesorbitan monolaurate (Tween 20) (0.5% v/v) (Sigma Chemical Co., Poole, U.K.), sodium azide (2% w/v) and RIA grade BSA (0.1% w/v) (Sigma Chemical Co., Poole, U.K.). The pH of the buffer was adjusted to 7.5 with sodium hydroxide (2 mol/l). Assay reagents decribed above were diluted with the assay buffer unless otherwise stated.

2.3. IGF-I Gene Expression

2.3.1 Reagents

Reagents used for RNA preparation procedures were purchased as guaranteed to be free of ribonucleases (RNases) where available, those that were not were treated with DEPC by adding it to solutions (0.1% v/v) for 24 hours followed by autoclaving. Water used in these procedures was also DEPC-treated. All Glassware was baked at 180°C for at least 2 hours to inactivate RNases and only disposable plasticware that had

not been handled was used. Restriction enzymes were obtained from Boehringer Mannheim U.K. Ltd. (Lewes, U.K.) unless otherwise indicated and the reactions were performed in buffers supplied by this company.

2.3.2. Tissue collection

Birds were killed by cervical dislocation. Liver and pre-ovulatory ovarian follicles (F1-F5) were removed and placed in a chaotropic solution of guanidinium isothiocyanate (4 mol/l) (Life Technologies Ltd., Paisley, U.K.), sodium citrate (25 mmol/l, pH 7), sarcosyl (0.5% w/v) and ß-mercaptoethanol (0.1 mol/l) (Fluka Chemicals Ltd., Glossop, U.K.). Pre-ovulatory ovarian follicles were first dissected in RNase-free sterile D-PBS as described above in Section 2.2.2.

2.3.3. RNA Preparation

The method of preparation used was based closely on that described by Chomczynski and Sacchi (1987). The tissues were homogenised in a chaotropic solution (see Section 2.3.2 above). The homogenate was passed by syringe through a needle several times in order to shear the DNA and then centrifuged at 400 g for 5 minutes to remove the cellular debris. Aliquots (0.5 ml) of the homogenates were then dispensed into 1.5 ml microcentrifuge tubes, then 50 µl of sodium acetate (2 mol/l, pH4), 500 µl of water-saturated phenol and 100 µl of chloroform : isoamyl alcohol mixture (49 : 1) were added to each tube (with mixing following each addition). Samples were cooled on ice for 30-60 minutes and centrifuged at 10,000 g for 20 minutes at 4°C after which each had separated into a non-aqueous phase and an aqueous phase containing the RNA. The latter was carefully removed with a Pasteur pipette to a clean tube. The RNA was then precipitated by adding an equal volume of cold isopropanol (propan-2-ol) and placing the sample in a deep freeze at -20°C for at least 1 hour. The RNA was pelleted by centrifugation at 10,000 g for 20 minutes, re-suspended in 300 µl of the chaotropic solution, and re-precipitated as described above. The pellet was washed with 70% ethanol, dried in a vacuum desiccator and then re-

suspended in water. An aliquot (5 µl) was taken, diluted to 1 ml with water and the optical densities at 260 and 280 nm measured to quantify the RNA and the contaminating protein, repecively, using a U.V. spectrophotometer. The sample was then diluted with water to a known RNA concentration and stored frozen at -80°C.

2.3.4. Reverse transcription-polymerase chain reaction

The polymerase chain reaction (PCR) is described in publications by Mullis and Faloona (1987) and Saiki *et al.* (1985; 1988). PCR allows amplification of DNA segments *in vitro* through a succession of incubation steps at different temperatures. Briefly, double stranded DNA is heat-denatured, two primers complementary to the 3' boundaries of the target sequence are annealed at low temperature then extended at an intermediate temperature. These three steps comprise a single PCR cycle.

RNA prepared as described in Section 2.3. was reverse transcribed to cDNA as follows: 2.5 µl DEPC-treated H_2O; 0.5 units (1 µl) RNA Block II (Stratagene Ltd, Cambridge, U.K.); 1 µl of 1 µg/µl acetylated BSA (Sigma Chemical Co.Ltd., Poole, U.K.); 2 µl 5 x RT Buffer; 1 µl Pd(N)6 (100 µg/ml in TE) (Pharmacia LKB, Milton Keynes, U.K.) and 1 µl RNA sample (1 µg/µl) were added, in the order shown, to a microcentrifuge tube. This mixture was incubated for 3 minutes in a hot block (65°C) and cooled immediately on ice: 1 µl dNTPs (5 mmol/l) and 0.5 µl M-MLV reverse transcriptase (200 units/µl) (Life Technologies Ltd., Paisley, U.K.) were then added. The reaction was incubated at room temperature for 10 minutes to ensure that the primers had annealed, then at 37°C for 1 hour in a water bath and finally at 95°C for 10 minutes to stop the reaction, this was then chilled on ice immediately. The resulting cDNA was used as a template for the PCR.

The PCR was set up with reagents supplied as a kit (Perkin-Elmer, Beaconsfield, U.K.) and following the protocols supplied with it. The reaction mixture contained the reverse transcribed cDNA, dNTPs, oligo primers, Taq polymerase and a buffer supplied with the kit. Volumes of reagents used are shown in Table 2.1. The total reaction volume was 50 µl.

Table 2.1. Reagents used in the polymerase chain reactions.

REAGENTS	VOLUMES (µl.)		
	β-actin	IGF-I	IGF-I + β-actin
H_2O	35	33.5	32.2
10 x PCR buffer	5	5	5
dNTPs (1.25 mmol/l)	8	8	8
5' oligo primer	8	8	8
3' oligo primer	0.65	0.8	0.8 + 0.65
Taq polymerase	0.65	0.65	0.65 + 0.65
cDNA	0.5	0.5	0.5
total	50	50	50

The reaction mixture (50 µl) was dispensed into autoclaved microcentrifuge tubes and covered with 100 µl of mineral oil (Sigma Chemical Co., Poole, U.K.) to reduce evaporation. The tubes were placed in a hot block with a programmable control system (Techne, Cambridge, U.K.) which was set to specify cycles of melting, annealing and extending temperatures and the times at which they were to be applied. The program also specified a longer extension period on completion of the set number of cycles in order to complete transcription in partly transcribed strands. On completion of the reaction the oil was removed from the reaction tubes by extracting twice with chloroform equilibriated in TE; tubes were vortexed and centrifuged on addition of 200 µl of chloroform, the bottom (oil) phase was then aspirated and discarded. A further 200 µl chloroform was added and, following further vortexing and centrifugation, the upper (aqueous) phase containing the DNA was transferred into new tubes.

In the experiments described in Sections 3.2.1, 3.2.2 and 3.2.4., DNA oligo-primers specific for both IGF-I and ß-actin were used. The expression of ß-actin measured using PCR was used as a positive control for the integrity of the RNA

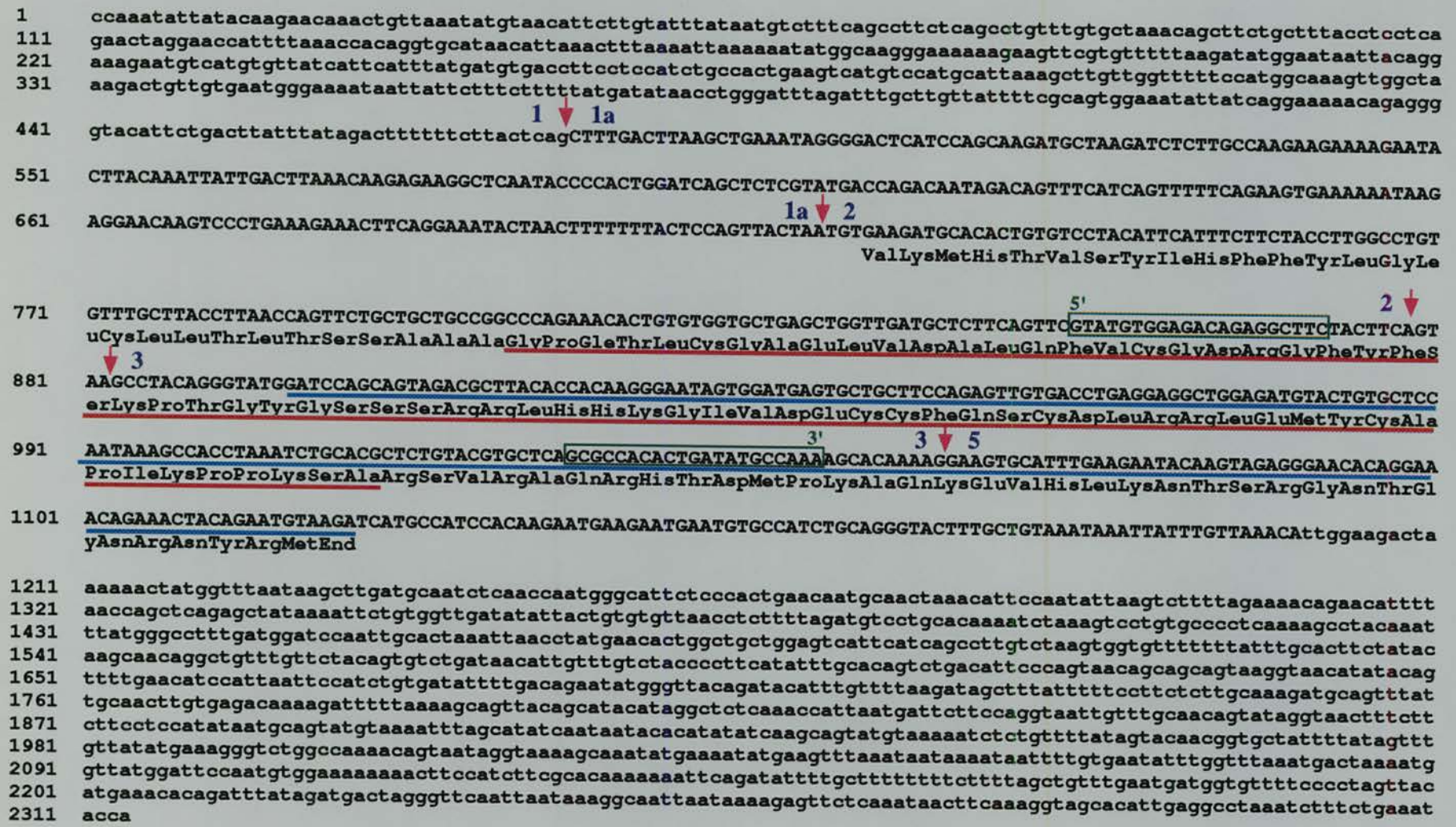

Figure 2.2 The sequence of chicken IGF-I cDNA clone 508 and the corresponding predicted amino acid sequence as described by Fawcett and Bulfield (1990). The amino acid sequence of mature chicken IGF-I is underlined in red. The boxed seqences are those of oligo primers used for PCR, the sequence underlined in blue is that of the cDNA probe used for Southern blots. Arrows indicate exon boundaries.

samples under investigation. Both sets of primers were chosen in order to avoid generation of primer-dimers i.e. complementarity at the 3' ends and palindromic sequences within primers. These synthetic oligo primers were made commercially (Oswel DNA, Edinburgh, U.K.). The IGF-I primer sequences are shown in Figure 2.3 below.

The IGF-I primers were complementary to 21 base sequences within the chicken IGF-I gene shown in Figure 2.2. The 5' sequence was complementary to the 3' end of exon 2 and the 3' sequence was complementary to the 3' end of exon 3. The cDNA which was predicted to be amplified with IGF-I primers in the PCR was 200 b.p. consisting of 28 b.p. of exon 2 and all of exon 3 except 10 b.p. at the 3' end. This sequence was chosen because it codes for the mature cIGF-I peptide (see Figure 2.2 above). An intron/exon boundary was also included in the sequence to be amplified so that amplification of any contaminating genomic IGF-I DNA would result in cDNA much greater in length than 200 b.p. and would therefore be easily identified. The predicted size of the PCR product produced with ß-actin primers was 290 b.p.

IGF-I 5'
5'- GTA TGT GGA GAC AGA GGC TTC -3'

IGF-I 3'
5'- TTT GGC ATA TCA GTG TGG CGC -3'

Figure 2.3. The DNA sequences of oligo primers specific for cIGF-I DNA.

To avoid contamination of reactions with DNA from other sources (reported as a problem by some workers), reactions were set up using positive displacement pipettes (which avoid cross-contaminating aerosols) in clean laminar flow cabinets.

2.3.5. Gel electrophoresis of PCR products

The products of the PCR reactions were analysed by two different methods of electrophoresis; vertical polyacrylamide gel electrophoresis (PAGE) and horizontal agarose gel electrophoresis. For PAGE, an acrylamide solution (5% w/v) was made with 5 ml 10x TBE buffer; 8.3 ml 30% acrylamide solution (29 : 1: 70, acrylamide : bis-acrylamide : water) and 36.7 ml water, 1x TBE is Tris-borate (89 mmol/l), boric acid (89 mmol/l) and EDTA (2 mmol/l, pH 8). The solution was de-gassed with a vacuum pump, then 160 µl of ammonium persulphate solution (10% w/v) and 160 µl of TEMED were added to the solution. The gel was cast on glass plates and allowed to set for 15 minutes, following which the casting comb was removed and the wells were washed out with 1x TBE. Samples were loaded and run at 100 V for 2-3 hours or at 20 V overnight in 1x TBE. Following electrophoresis the gel was stained with ethidium bromide and then photographed while transilluminated with U.V. light (nucleic acids stained with ethidium bromide fluoresce in these conditions).

For the second method, PCR product samples were mixed 1 : 6 with a gel loading buffer (bromophenol blue (0.25% w/v), xylene cyanol (0.25% w/v) and glycerol (30% v/v) in DEPC-treated water), loaded on 4% NuSieve GTC agarose gels (ICN Biomedicals Ltd., High Wycomb, U.K.) with DNA size markers and run submerged in 1x TAE buffer (Tris acetate (40 mmol/l), EDTA (1 mmol/l, pH 8)) at 50 V for 4-5 hours. Following electrophoresis the gel was stained, photographed and bands measured as described above.

2.3.6. Southern analysis

Agarose gels were soaked and agitated in a denaturation buffer (sodium hydroxide (0.5 mol/l); sodium chloride (1.5 mol/l)) for 20 minutes; this was repeated with fresh denaturation buffer and then twice with a neutralising buffer (Tris-HCl (1 mol/l); sodium chloride (1.5 mol/l)). Filters were cut to the size of the gels from Hybond-N hybridising membrane (Amersham International plc, Amersham, U.K.), soaked in 2x SSC for 20 minutes and placed on the gel. The gel was placed on a wick

of blotting paper fed from a reservoir of 10x SSC below. The ensemble was then covered with absorbant blotting material and left for at least 16 hours to blot by capillary action. Following blotting the filter membrane was rinsed in 2x SSC in order to remove adhering agarose. The nucleic acids were fixed to the filter by exposing it to U.V. light in a Stratalinker.

Filters were pre-hybridised at 37°C for 3 hours in 25 ml of a solution containing de-ionised formamide (50% v/v), 20 x SSC (25% v/v), 100 x Denhardt's solution (RIA grade BSA (2% w/v), Ficoll (2% w/v)(Pharmacia LKB Ltd., Milton Keynes, U.K.) and polyvinylpyrrolidone (2% w/v)) (5% v/v), SDS (0.5% w/v), dextran sulphate (3% w/v) and sonicated salmon sperm (4% v/v). For hybridisation the solution was replaced with a similar solution which did not contain sonicated salmon sperm. The [^{32}P]-cDNA chicken IGF-I probe, made as described below, was added.

A 224 b.p. chicken IGF-I cDNA fragment was cut with a *Sau*3a restriction enzyme digest from the IGF507 clone (Fawcett and Bulfield, 1990), the sequence is indicated on Figure 2.2. The [^{32}P]-cDNA probe was made (using this fragment as a template) with a random primed DNA labelling kit (Boehringer Mannheim U.K. Ltd., Lewes, U.K.). The DNA (25 ng) was boiled for 10 minutes, aliquoted into a microcentrifuge tube then the following reagents from the kit were added; 3 µl dATP dGTP dTTP mixture (1:1:1), 2 µl hexanucleotide solution, 1 µl Klenow enzyme and 5 µl [^{32}-P]-αdCTP (Amersham International plc, Amersham, U.K.). The reaction mixture was then incubated at 37°C for 30 minutes and the reaction stopped by adding 2 µl EDTA (0.2 mol/l, pH 8.0). The probe was diluted with autoclaved TE to 200 µl, boiled for 5 minutes and placed on ice immediately prior to use.

Hybridisation was performed for approximately 16 hours at 37°C. Both the pre-hybridisation and hybridisation steps were carried out in sealed perspex hybridisation chambers, placed on a shaker. Following hybridisation the filter was rinsed with 2x SSPE, SDS (0.1% w/v). It was then washed in the same solution for 2x 20 mins (at 65°C) followed by washes in 1x SSPE, SDS (0.1% w/v) then 0.1x SSPE, SDS (0.1% w/v) at the same temperature. Filters were air dried and placed in cassettes with X-ray

film. Exposures were usually for 16 hours at -70°C but the time varied according to the filter activity. Exposed films were developed with an automated X-ray film developing machine.

2.4.TISSUE CULTURE

2.4.1. Reagents

The medium used for cell culture was medium 199 (M199) containing Earle's salts and sodium bicarbonate, supplied as a sterile filtered solution. This was supplemented with HEPES buffer (20 mmol/l), L-glutamine (2 mmol/l), sodium pyruvate (2 mmol/l), streptomycin (0.1 mg/ml) and penicillin (1000 u/ml) before use in the culture systems described below. The collagenase used had been purified from *Clostridium histolyticum*. The serum used was fetal calf serum (FCS), this was heat inactivated by heating to 56°C for 30 minutes prior to use. All of the reagents listed above were obtained from Sigma Chemical Co.(Poole, U.K.).

2.4.2. Coating tissue culture plates

In some experiments, the tissue culture plates were coated with substrates as described in the following sections.

2.4.2.1. Collagen treatment

A solution of collagen type I (2.5 µg/ml) from calf skin, (Sigma Chemical Co., Poole, U.K.) was prepared with sterile acetic acid (0.1 mol/l) according to manufacturers instructions. Multiwell tissue culture dishes were coated with 250 µl collagen solution per 1 cm^2 well and the dishes were dried in a laminar flow hood. The wells were then rinsed with sterile distilled water in order to remove the acid prior to use.

2.4.2.2. Gelatin treatment

A solution of gelatin in water (0.1% w/v) was prepared and filtered into a sterile bottle. This solution was autoclaved and used to coat wells (250 µl per 1 cm^2 well) using the methods described above (there is no requirement to rinse the wells).

2.4.2.3. FCS treatment

Wells were coated with 250 µl heat-inactivated FCS and incubated at 37°C in a humidified incubator overnight. The FCS was aspirated from the wells prior to use.

2.4.3. Tissue collection

Pre-ovulatory ovarian follicles were removed from the ovary immediately after the hen was killed and placed in sterile, Dulbecos phosphate-buffered saline (D-PBS) at room temperature. Follicles were then transferred to a laminar air-flow tissue culture cabinet. The follicles were dissected as described in Section 2.2.2.under sterile conditions. The dissected tissues were placed in separate sterile 30 ml Universal tubes containing supplemented medium M199. The dissection procedure is shown in Figure 2.1.

2.4.4. Cell dispersal

2.4.4.1. Granulosa cells

Granulosa cell layers were placed in sterile plastic petri dishes where they were minced with fine scissors and transferred by pipette to a sterile 30 ml plastic Universal vial containing 1 ml of a 1 mg/ml type I collagenase solution (in supplemented medium M199). The cells were digested in this solution for 5 minutes at room temperature, aided by repeatedly drawing in and aspirating the partially digested suspension with a pipette. The cell suspension was then diluted to a volume of 5 ml with supplemented medium M199 and pelleted by centrifugation at 300 g for 10 minutes, the supernatant was discarded and the cells re-suspended in 5 ml of fresh

supplemented medium M199. The cells were washed twice more by this procedure in order to remove the collagenase.

2.4.4.2. Thecal cells.

Thecal cell layers were placed in sterile plastic petri dishes and minced as described in Section 2.4.4.1. above. They were then transferred to a sterile 30 ml Universal vial containing 1ml of a 5 mg/ml collagenase solution This vial was submerged in a shaking water bath at 37°C and the cells were digested for 90 minutes, assisted by pipetting (as described above in Section 2.4.4.1.) every 15 minutes. The volume of the suspension was then made up to 5 ml with supplemented medium M199 and percoll (Sigma Chemical Co., Poole, U.K.) to produce a 40% percoll solution (v/v); this was then mixed and centrifuged at 400 g for 20 minutes. The gradient separated the thecal cells (at the top) from the red blood cells (RBCs) which were in the pellet; the top layer containing thecal cells was carefully removed to a sterile 30 ml universal vial and medium M199 added to produce a solution with less than 20% percoll (v/v). Thecal cells were then pelleted by centrifugation at 300 g for 10 minutes and the pellet re-suspended in 1 ml lysing buffer (ammonium chloride (0.13 mol/l), Tris (17 mmol/l), potassium hydrogen carbonate (10 mmol/l), pH 7.5) for 2-3 minutes in order to lyse any remaining RBCs. Supplemented medium M199 was then added to make up the volume to 5 ml and the cells were pelleted and washed as described in Section 2.4.4.1. Microscopic examination of the dispersed cells showed that no RBCs were present in the preparation.

2.4.5. Cell culture

The cell density and viability of cell preparations was measured by the trypan blue exclusion test. Viable cells do not take up and, therefore, are not stained with the dye; cells with damaged cell membranes are stained. Therefore, by measuring the proportion of unstained to total cells the cell viability of the particular preparation can be calculated. Equal aliquots of the cell preparations and the trypan blue dye (ICN

Biomedicals Ltd., High Wycombe, U.K.) were mixed. This suspension was loaded on an improved Neubauer haemocytometer, depth 0.1 mm and smallest square 1/400 mm^2 (FSA Laboratory Supplies, Loughborough, U.K.). The total cell density and the percentage cell viability was measured and from these values the total viable cell density calculated. Cell viability of both cell types was always greater than 90% and usually greater than 95%. All cell densities quoted in subsequent sections are for viable cells. Cells were plated on sterile multiwell plastic tissue culture plates (Costar U.K. Ltd, High Wycombe, U.K.). During manufacture, these plates were subjected to corona discharge; this process involves subjecting them to very high voltages in a controlled atmosphere resulting in the removal of oxygen and the production of a charged surface. The plates were also subjected to radiation to render them sterile. Both of these processes enhance cell attachment to the plastic surfaces. Plates were used as supplied, or treated with serum, fibronectin, collagen or gelatin (as described in Section 2.4.2.). Cells were cultured in a humidified CO_2 incubator, with an atmosphere containing 5% CO_2 and at a temperature of 41°C (avian body temperature).

In experiments involving anti-LH antisera, the antibodies were added in sufficient quantity calculated to bind at least twice the amount of LH used. Final concentrations of 1 : 1000 for anti chicken LH (3/9) and of 1 : 5000 for anti ovine LH were used to immunoneutralize chicken and ovine LH , respectively, added to the culture media at a concentration of 25 ng/ml.

2.4.6. Immunocytochemistry

Cells were prepared for culture as described in Sections 3.3.1 and 3.3.2. Granulosa and thecal cells were plated on autoclaved glass coverslips in 12-well multiwell plates at 50,000 and 250,000 cells per cm^2 respectively in 1 ml medium M199 with FCS (3% v/v). Cells were allowed to attach in a 48 hour period after which they were washed with serum-free medium M199 then stained.

The principles of the immunocytochemical method used to determine IGF-I expression in these cells are shown in Figure 2.4. The primary antiserum was raised in

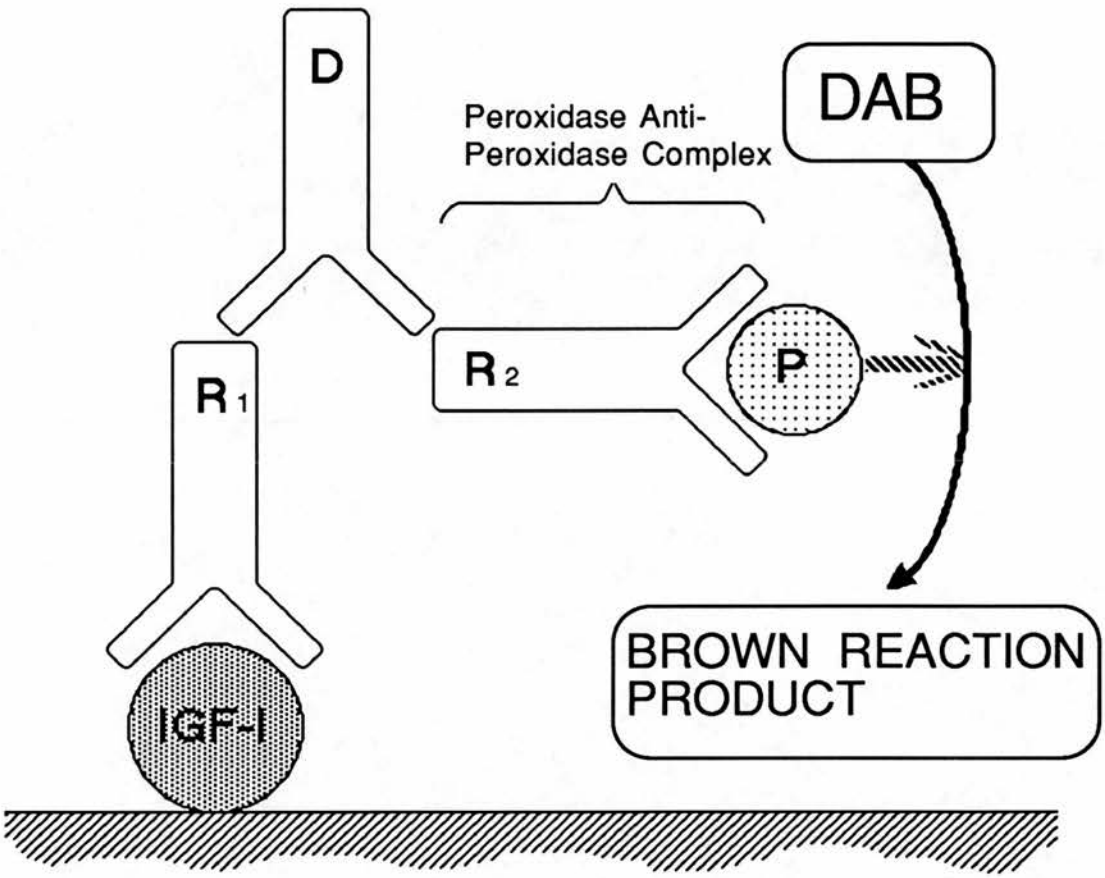

Figure 2.4. The immunocytochemical staining process used to detect IGF-I. The primary antiserum (R1) binds to IGF-I and is then bound to the second antiserum (D), donkey anti-rabbit. The rabbit peroxidase anti-peroxidase is then bound to this complex by the second antibody. The complex is visualised when 3,3' Diaminobenzidine (DAB) is added, the peroxidase (P) oxidises the DAB substrate to a brown product.

rabbits and the second antiserum (which binds to the primary antiserum) was raised in donkey against rabbit immunoglobulins. The primary antibody binds IGF-I, the second (bridging) antibody is added in excess and binds the primary antibody and the rabbit peroxidase anti-peroxidase complex. The addition of 3, 3'-diaminobenzidine (DAB) results in a complex reactionof this substrate with peroxidase, the end point of which is a development of brown colouration. The chemistry of the oxidation of DAB has not been clearly established, partly because the reaction product is so highly insoluble and, therefore, difficult to work with (Karnovsky and Robinson, 1981).

Cells were fixed in 4% paraformaldehyde for 1h at room temperature, washed for 15 minutes in PBS (0.1 mol/l) (repeated x3). Cells were then incubated for 60 minutes in H_2O_2 (1% v/v), 10 minutes in saponin and 30 minutes in NDS (5% v/v) and were washed as described above with PBS between each incubation. The primary antibody was incubated with the cells at 4°C overnight which were then incubated with donkey anti rabbit serum (DARS), the second antibody, used at 1:200 for 1hour at room temperature and with rabbit peroxidase-anti-peroxidase (PAP) used at 1:150 for 1hour at room temperature; cells were washed in PBS following each incubation. The cells were then washed with Tris-HCl (50 mmol/l, pH 7.4) for 5 minutes at room temperature then with 10 ml of a DAB solution (DAB (0.05% w/v), Tris-HCl (50 mmol/l, pH 7.4), H_2O_2 (0.1% v/v)) was added to stain the antibody complex. At this point the cells were photographed on an inverse microscope using Kodak Ektachrome 64T slide film. Following photography the cells were washed twice in Tris-HCl (50 mmol/l, pH 7.4), then dehydrated with alcohol followed by xylene and mounted with DPX on glass slides.

2.4.7. Cell growth

2.4.7.1. Measurement of [^{3}H]-thymidine incorporation

In order to measure DNA synthesis, the incorporation of a tritiated [^{3}H] species of the nucleoside, thymidine (a constituent of DNA) into the DNA of cells was used as

an index of this activity. This radioisotope was obtained from Amersham International plc (Amersham, U.K.).

Cells suspended in medium M199 supplemented as described in Section 2.4.1. were plated in accurately measured volumes with a pipette and then placed in the incubator, undisturbed for 24 hours; this was the attachment period during which any movement or fluctuation in temperature or CO_2 inhibited attachment. Cells were visually inspected using a phase-contrast microscope after 24 hours and every 24 hours thereafter until termination of the culture. After 48 hours the original medium was replaced with fresh supplemented medium M199 without any FCS in order to starve the cells and remove serum which may otherwise interfere with cell growth measurements. The cells were growth restricted in order to synchronise them in G0. After 72 hours the medium was replaced and supplemented with the experimental treatments to stimulate cell growth, 8 hours following this (80 hours) [^{3}H]-thymidine label (Amersham International plc, Amersham, U.K.) was added to each well (0.3 μCi/ml). The culture procedure was terminated after 96 hours by aspirating the medium completely, taking care not to disturb the monolayer of cells. Wells were washed twice with unsupplemented medium M199 (2 x 500 μl per well) warmed to room temperature, then 1 ml of cold trichloroacetic acid (10% w/v) was added to each well and the plates incubated at 4°C for 20 minutes to precipitate the DNA. The acid was aspirated and 500 μl of sodium hydroxide (0.5 mol/l) added per well, then the plates were incubated at 37°C for 2 hours on a shaker to solubilise the attached cells. The solution from each well was transferred to scintillation vials which were filled with Optiphase X scintillant (Pharmacia LKB, Milton Keynes, U.K.). The radioactivity in the samples was then measured in a scintillation counter.

2.4.7.2. Measurement of DNA

The DNA content of cultured granulosa and thecal cells was measured using the fluorometric assay described by West *et al.* (1985).

DNA standards were set up in test tubes by diluting a stock solution of salmon testis DNA (2 mg/ml) (Sigma Chemical Co., Poole U.K.), the final concentrations of these were 100, 200, 400, 600, 800, 1000, and 2000 ng/tube. The cells and standards were treated with 1.4 ml cold EDTA (10 mmol/l, pH 12.3), incubated at 37°C for 20 minutes and then cooled on ice. The pH was then lowered to 7 with potassium dihydrogen orthophosphate (2 mol/l). Bisbenzamide (1.5 ml, 200 ng/ml) (Hoescht No. 33258, supplied by Sigma Chemical Co., Poole, U.K.) was added to all the samples and the resulting fluorescence was measured by spectrophotometry: the excitation and emission wavelengths were 350 and 455 nm, respectively.

2.4.8. Steroidogenesis

Oestradiol, progesterone and androstenedione were measured in cell-conditioned medium by radioimmunoassay. Samples were stored frozen at -20°C before assay.

2.4.8.1. Progesterone radioimmunoassay

The progesterone assay was a double antibody RIA previously described by Corrie *et al.* (1981) and Webb (1987). The primary antiserum was produced in a rabbit (code R 31/8, obtained from Dr. R. Webb, IAPGR, Roslin, U.K.)

The reagents were dispensed into glass tubes and diluted with phosphate gelatin buffer containing *di*-sodium hydrogen orthophosphate (50 mmol/l) sodium chloride (0.15 mol/l) and thimerosal (0.1% w/v) at pH 7.5. The progesterone standard curve was constructed at concentrations of 10, 20, 40, 70, 130, 250, 500 and 1000 pg/tube. The label used was [^{125}I]-progesterone (obtained from Ian Swanson, MRC Centre for Reproductive Biology, Edinburgh). The primary antiserum (R 31/8) was used at an initial dilution of 1 : 8000. The secondary antiserum was DARS used at an initial dilution of 1 : 35 (diluted with PBS containing EDTA (10 mmol/l)), the carrier was NRS used at a dilution of 1 : 300.

The standards and samples were diluted with assay buffer (to 500 µl), the label was added (100 µl) then the primary antiserum (200 µl). Tubes were then vortexed and incubated for 3 hours at room temperature. The secondary antiserum (100 µl) and carrier (100 µl) were added, the tubes re-vortexed and incubated overnight at 4°C. The tubes were washed with 1 ml of buffer and spun at 1860 g for 30 minutes; the supernatant was discarded and the radioactivity in the pellets measured on a Gammamaster gamma counter.

2.4.8.3. Androstenedione radioimmunoassay

An androstenedione radioimmunoassay kit was purchased from Biogenesis (Bournemouth, U.K.). The kit included a label ([^{125}I]-androstenedione), standards (final concentrations of 1, 3, 10, 30, 100, 300, 1000 and 3000 pg / tube), primary antibody (rabbit anti-androstenedione serum) and a secondary antibody (goat anti-rabbit gamma globulin serum). Precipitation of the antibody-antigen complex was aided with polyethylene glycol. The antiserum supplied was diluted 1 : 3 prior to use.

Samples (150 µl) or standard were aliquoted into glass test tubes in duplicate. To each tube 300 µl of label (approx. 20,000 c.p.m.) and 100 µl of the antiserum was added. The tubes were then vortexed and incubated at 37°C in a water bath for 30 minutes. The precipitating reagent (750 µl) was added and the tubes centrifuged at 1500 g at 4°C for 20 minutes. The supernatant was then aspirated and the radioactivity in the pellets measured on a Gammamaster gamma counter.

2.4.9. IGF Binding protein analysis

Cell-conditioned medium was concentrated using Microsep centrifugal micro-concentrators (Flowgen Instruments Ltd., Sittingbourne, U.K.), following the manufacturer's protocol. The concentrators had a 10 kDa molecular weight cut off and the final concentrated volume was 50 µl. The concentrate was denatured by boiling for 5 minutes in Laemlli buffer (Tris-HCl (50 mmol/l, pH 6.8), SDS (1% w/v), glycerol (10% (v/v) with bromophenol blue) prior to loading on an SDS polyacrylamide gel

with protein molecular weight markers (Sigma Chemical Co., Poole, U.K.). A 12% acrylamide separating gel was cast with a 5% stacking gel. Samples were run through the stacking gel at a current of 40 mA/gel. Once the samples were stacked the current was increased to 60 mA/gel. The running buffer used was Tris base (0.3% w/v), glycine (1.44% w/v) and SDS (0.1% w/v) After electrophoresis the gel was carefully placed on a sheet of nitrocellulose and electroblotted at 120 mA; transfer was complete in 1-2 hours.

Following electroblotting, the nitrocellulose filters were placed in chambers containing TS buffer (Tris HCl (10 mmol/l, pH 7.4), sodium chloride (0.15 mol/l)) with sodium azide (0.5 mg/ml) and Nonidet P40 (3% v/v) and incubated for 30 minutes at 4°C. The filters were then rinsed with TS buffer and incubated with TS buffer containing RIA grade BSA (1% w/v)(Sigma Chemical Co., Poole, U.K.) for 2 hours. This was followed by incubation for 2 periods of 20 minutes with TS buffer containing tween 20 (0.1% v/v) then an overnight (minimum 16 hours) incubation with TS buffer containing RIA grade BSA (1% w/v), Tween 20 (0.1% v/v) and [^{125}I]-IGF-I (200,000 cpm/ml) with or without unlabelled IGF-I (2 μg/filter, 0.2 μg/ml). The filter was then washed in TS buffer containing Tween 20 (0.1% v/v) (2 x 15 mins.) and then in TS buffer (2 x 15 mins). All incubations and washes were carried out at 4°C. The filter was air dried and placed in a cassette with X-ray film for autoradiography for approximately 7 days at -70°C. The film was developed in an automated film developing machine.

2.4.10. Ligand binding studies

The method used has been previously described (Ewton *et al.*, 1987; Duclos *et al.*, 1991). Tissue culture plates were pre-incubated overnight with a solution of RIA grade BSA (10% w/v) (Sigma Chemical Co., Poole, U.K.) to reduce non-specific binding of the radiolabelled ligand to the surface of the wells. This solution was aspirated immediately prior to cell plating. Ovarian cells were prepared for culture and plated as described in Sections 2.4.4. and 2.4.5. Cells were seeded for 48 hours in

medium M199 with FCS (3% v/v) then growth restricted for 24 hours in serum-free medium M199. This medium was replaced with medium M199 containing 0.5% BSA. Treatments were then added to the wells and the plates incubated at room temperature on a slowly rotating shaker for 4 hours. Medium was aspirated and the cells washed twice in cold medium M199. Cells were solubilised in SDS (0.2% w/v) on a rotary shaker for 2-4 hours at room temperature. Dissolved cells were dispensed into polystyrene tubes and the radioactivity measured on a Gammamaster gamma counter.

2.5. DATA ANALYSIS

Data from replicate experiments were meaned and the standard devaiation from the mean calculated for each replicate group. Student's t test was used to test significance between groups of data. Analysis of variance was performed on data using MINITAB (Minitab, Inc.) in order to determine whether two treatments were synergistic or not (an example of this analysis is shown in Section 2.5.1. below).

The AssayZap program (Biosoft, Cambridge, U.K.) was used to analyse and plot the data obtained from the radioimmunoassays described in 2.2.3 and 2.4.7. The ALLFIT program (De Lean *et al.*, 1978) was used to calculate ED50s of dose-response curves and for comparisons of curves, the LIGAND program (Munson and Rodbard, 1980) was used to fit the binding data generated in the receptor binding studies described in Chapter 6.

2.5.1. Determination of synergy

An example of the data analysis performed to determine the occurrence of synergy is shown below. In this example the data set represents the results of an experiment in which granulosa cells were treated with IGF-I, LH or IGF-I with LH and then the effects on [^{3}H]-thymidine incorporation by these cells was measured (values are c.p.m./well).

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AUG. 27, 1991 *** STORAGE AVAILABLE 38000

MTB >      READ  C1  C2  C3
DATA>      0     0     556.1
DATA>      0     0     551.2
DATA>      0     0     549.4
DATA>      1     0     1430.1
DATA>      1     0     1205.8
DATA>      1     0     1148.8
DATA>      0     1     1275.8
DATA>      0     1     1233.6
DATA>      0     1     1121.2
DATA>      1     1     3967.5
DATA>      1     1     3517.5
DATA>      1     1     3489.1
DATA>      END
     12  ROWS READ

MTB >      ANOVA  C3= C1+C2+C1*C2
```

Factor	Type	Levels	Values
C1	fixed	2	0 1
C2	fixed	2	0 1

Analysis of Variance for C3

Source	DF	SS	MS	F	P
C1	1	7475776	7475776	297.42	0.000
C2	1	6997172	6997172	278.38	0.000
C1*C2	1	2266787	2266787	90.18	0.000
Error	8	201083	25135		
Total	11	16940818	1540074		

CHAPTER 3: THE EXPRESSION OF IGF-I mRNA AND THE OCCURRENCE OF IGF-I IN CHICKEN FOLLICULAR TISSUES

3.1. INTRODUCTION

IGF-I gene expression and peptide concentrations were measured in the granulosa and thecal tissue of chicken ovarian pre-ovulatory follicles. RNA was extracted from homogenised tissue samples and the IGF-I messenger (m) RNA content analysed using a method involving the reverse transcription polymerase chain reaction (RT-PCR) in which RNA was reverse transcribed to cDNA, amplified with IGF-I-specific primers and the resulting DNA analysed for IGF-I cDNA. The concentrations of IGF-I peptide in these tissues was examined by extracting tissue homogenates and then measuring the immunoactive IGF-I activity in the extracts by radioimmunoassay. The occurrence of IGF-I on granulosa and thecal cells *in vitro* was examined by immunocytochemistry.

3.2. IGF-I GENE EXPRESSION IN FOLLICULAR TISSUES USING RT-PCR

3.2.1. Optimising PCR conditions

Polymerase chain reaction conditions were optimised in an experiment in which the annealing temperature and melting and extension times were varied. Total liver RNA was reverse transcribed and subjected to reactions with annealing temperatures from 52°C to 54°C. Melting and extension temperatures were 94°C and 72°C, respectively. The melting times were between 1 and 2 minutes and those for extension were 3 to 5 minutes. Products of the reaction were analysed by polyacrylamide gel electrophoresis (PAGE). The gels on which the IGF-I cDNA bands were clearest and without extraneous bands were obtained with an annealing temperature of 53°C and with melting and extending times of 1 minute and 3 minutes, respectively. These conditions were used in the experiments described below.

3.2.2. IGF-I gene expression in granulosa and thecal tissues

Each study was performed with RNA extracted from granulosa layers pooled from the F1, F2, F3 and F4 follicles of 3 laying hens and from similarly pooled thecal layers. RNA from liver was used as a positive control, since the liver has been shown to express IGF-I mRNA in chickens (Fawcett and Bulfield, 1990), distilled water was used as the negative control.

RNA from the three tissues and an equivalent volume of distilled water was reverse transcribed and the resulting cDNA (transcribed from 0.1 μg of RNA) was used for PCRs. Two sets of oligo primers were used; IGF-I primers with a predicted product size of 200 bp, and β-actin primers with a predicted product size of 290 bp. The second set of primers were used to control for the integrity of the RNA samples.

PCR products were subjected to PAGE. Analysis of the gels (shown in Figure 3.1a), showed that IGF-I cDNA and β-actin cDNA was present in samples derived from granulosa, theca and liver RNA, neither was present in the negative control.

3.2.3. Southern analysis of PCR products

The experiments described above were repeated. The resulting PCR products were run on 4% agarose gels, which were Southern blotted with a cIGF-I cDNA probe (Figure 3.1b).

Careful examination of the gel revealed bands of DNA of 200 bp and 290 bp in length, corresponding to the size of PCR reaction products expected for IGF-I cDNA and β-actin cDNA respectively. No bands were visible in the negative control tracks. On the Southern blot the probe hybridised with DNA bands in the granulosa, theca and liver sample tracks and did not hybridise with anything in the

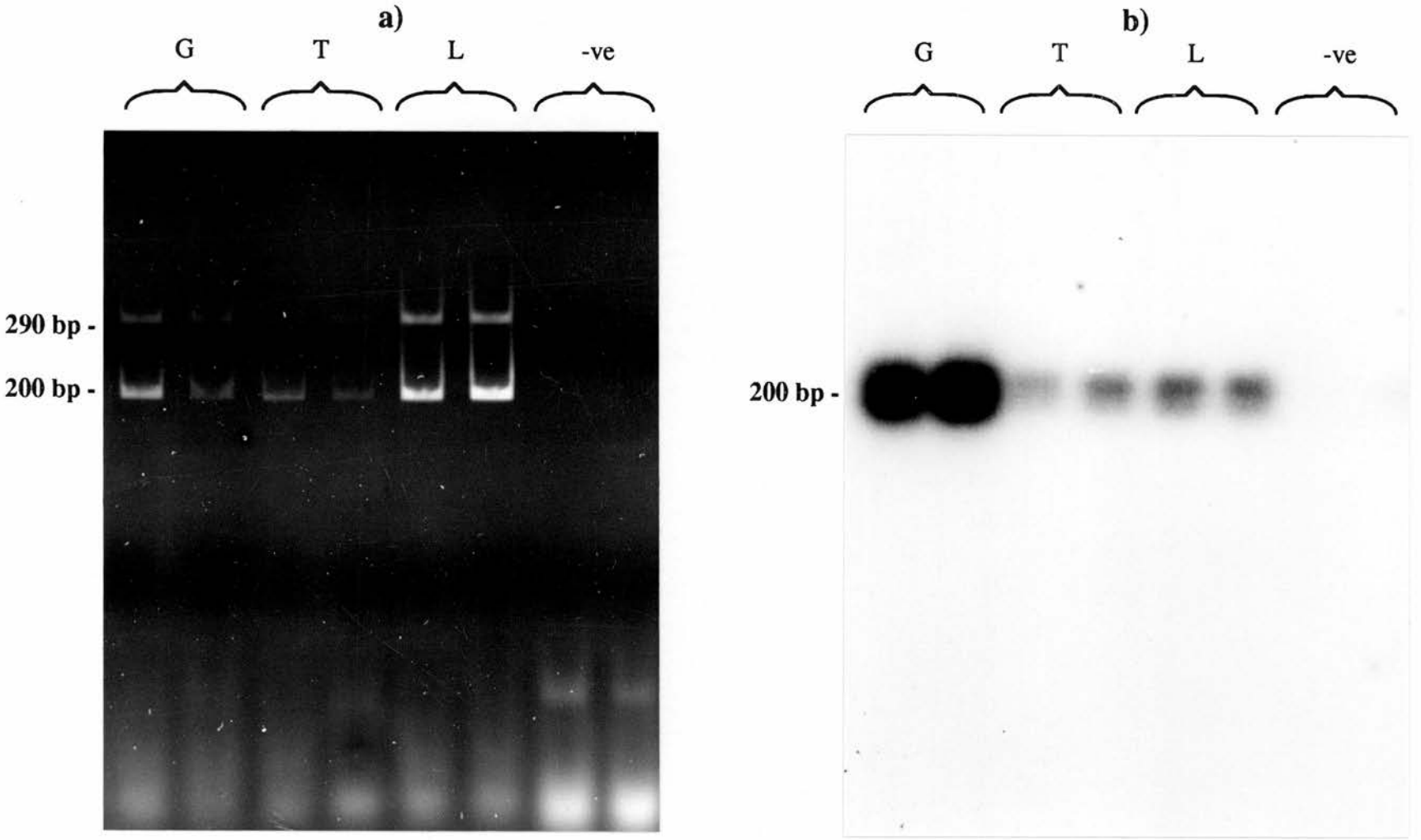

Figure 3.1 Photographs of (a) a PAGE gel of PCR products derived from granulosa (G), theca (T), liver (L) and distilled water negative control (-ve) then amplified with cIGF-I-specific primers; and (b) a Southern blot of the same samples run on an agarose gel.

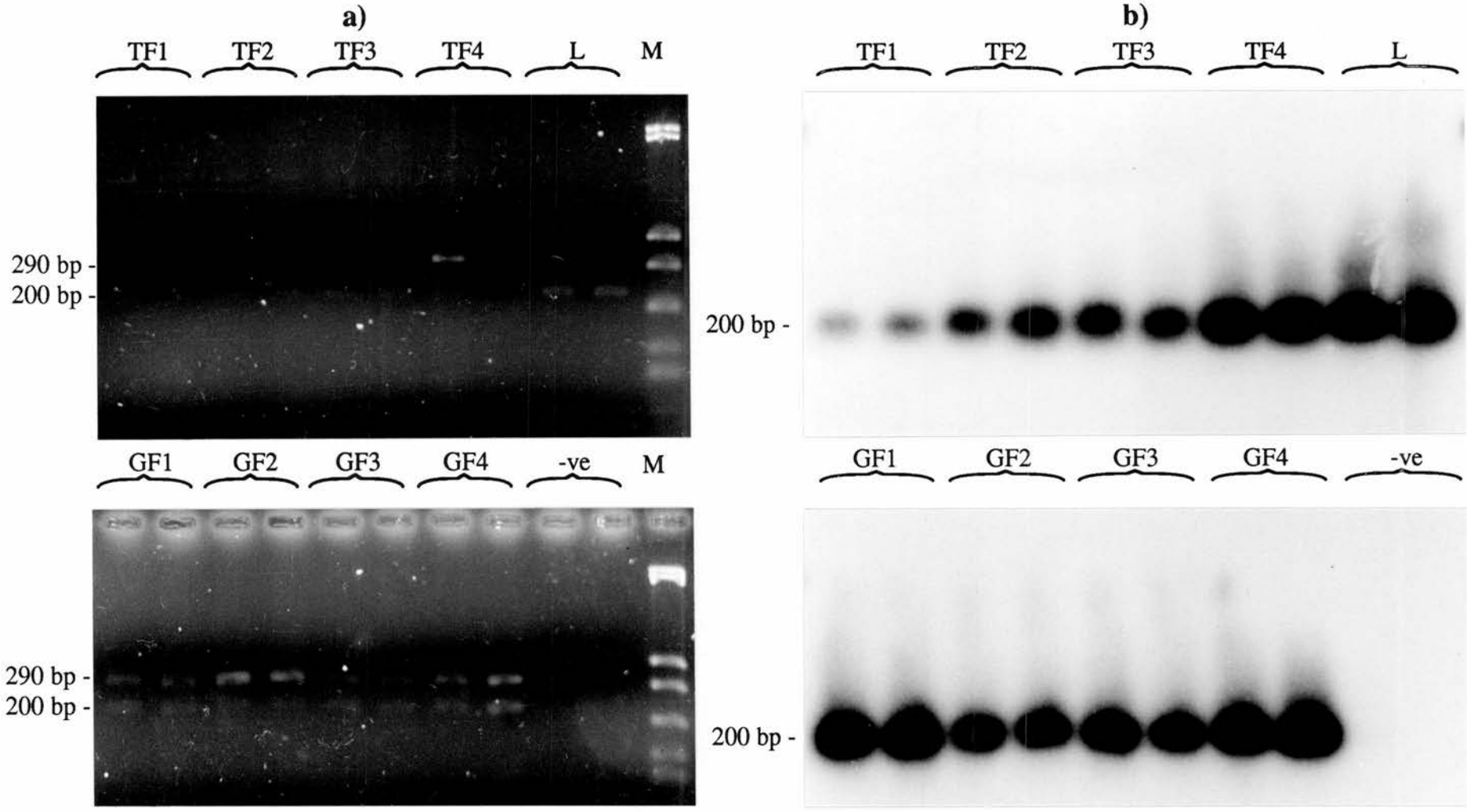

Figure 3.2 Photographs of a) agarose gels of PCR products derived from theca (T), liver (L) granulosa (G) and the distilled water negative control (-ve) then amplified with cIGF-I-specific primers and b) Southern blots of the same gels. The develop-mental stage of the follicles from which the RNA was extracted is indicated by F1-F4. DNA size markers (M) were used. The sizes of the expected cDNA products for the IGF-I and β-actin reactions were 200 bp and 290 bp, respectively.

negative control tracks. The hybridised DNA bands were 200 bp in length corresponding with the size of the IGF-I PCR reaction product. This is further confirmation that the bands of 200 b.p. are cIGF-I cDNA.

3.2.4. IGF-I gene expression in follicles at different stages of development

RNA was extracted from granulosa and thecal tissues of F1, F2, F3 and F4 follicles in the developmental hierarchy. These samples, together with liver RNA and distilled water as positive and negative controls, were analysed by RT-PCR and Southern blotting as described above. Photographs of the gels and the resulting autoradiograph are shown in Figure 3.2.

DNA bands of 200 bp (cIGF-I) and 290 bp (β-actin) in length were visible on sample tracks from follicles at all four developmental stages. The 200 b.p. band was confirmed as cIGF-I cDNA by the Southern blot. The degree of hybridisation intensity of the IGF-I probe to the cDNA derived from thecal RNA was, in ascending order, F1 < F2 < F3 < F4. However, when the experiment was repeated (results not shown) there was no detectable difference in the intensity of the hybridisation.

3.3. The Measurement Of IGF-I Peptide Concentrations In Chicken Follicular Tissues

IGF-I peptide concentrations in both granulosa and thecal tissues were measured. IGF-I was extracted from these tissues by two different methods and then measured by RIA.

3.3.1. Analysis of extracted tissue samples

Granulosa, theca and liver were removed from four laying hens; the material from each hen was kept separate. Samples of each tissue (1 g) and 1 ml of distilled water were extracted with acetic acid : ethanol : acetone (liver and distilled water were

included as positive and negative controls, respectively). IGF-I concentrations were measured by RIA. The results of this experiment and a repeat are shown in table 3.1.

Table 3.1 IGF-I concentrations in liver, granulosa and thecal tissue samples extracted with acetic acid, ethanol and acetone. In both experiments n = 4; * denotes $p > 0.001$ versus the other tissues in the experiment. Measurement was by radioiummunoassay.

TISSUE	IGF-I (NG/G WET WEIGHT ± S.E.M.)	
	Experiment 1	Experiment 2
Liver	1.93 ± 0.09	1.60 ± 0.38
Granulosa	6.42 ± 0.09 *	3.00 ± 0.03 *
Theca	2.80 ± 0.17	2.06 ± 0.02

IGF-I was present in all the tissues examined in these experiments, the greatest concentrations were found in the granulosa samples in both experiments. However, concentrations of IGF-I in the second experiment were lower. The next highest concentration was found in the theca then the liver. IGF-I was undetected in the negative controls in both experiments.

3.3.2. Analysis of acid chromatographed tissue samples

IGF-I extraction methods have been criticised for incomplete removal of the binding proteins from this peptide, a potential source of interference. In an attempt to confirm the results described above, similar tissues were subjected to acid chromatography to separate IGF-I more completely from binding proteins.

3.3.2.1. Column characterisation

A chromatography column was set up and characterised as described in Chapter 2. This first peak was eluted from the column at a volume of 18 ml, this

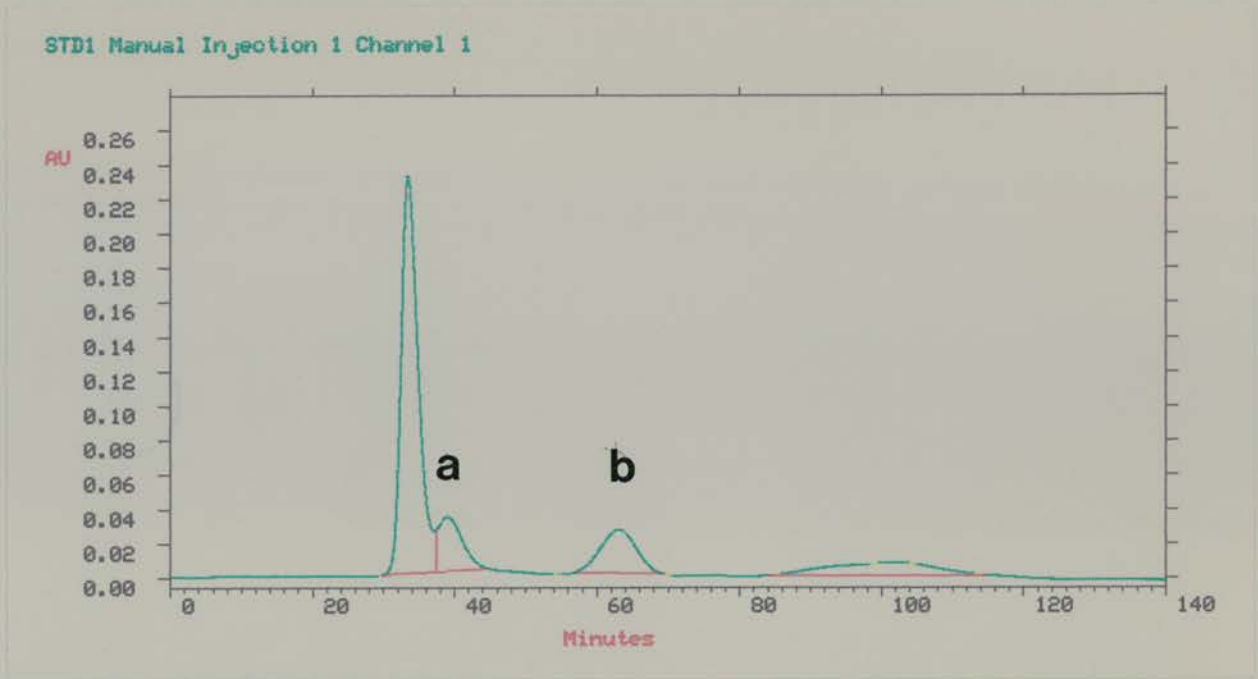

Figure 3.3. U.V. absorbance of marker proteins run on a Sephadex G 50-superfine column used to separate IGF-I from its binding proteins. The column flow rate was 0.55 ml per minute. Cytochrome-C and aprotinin peaks are marked as (**a**) and (**b**) respectively.

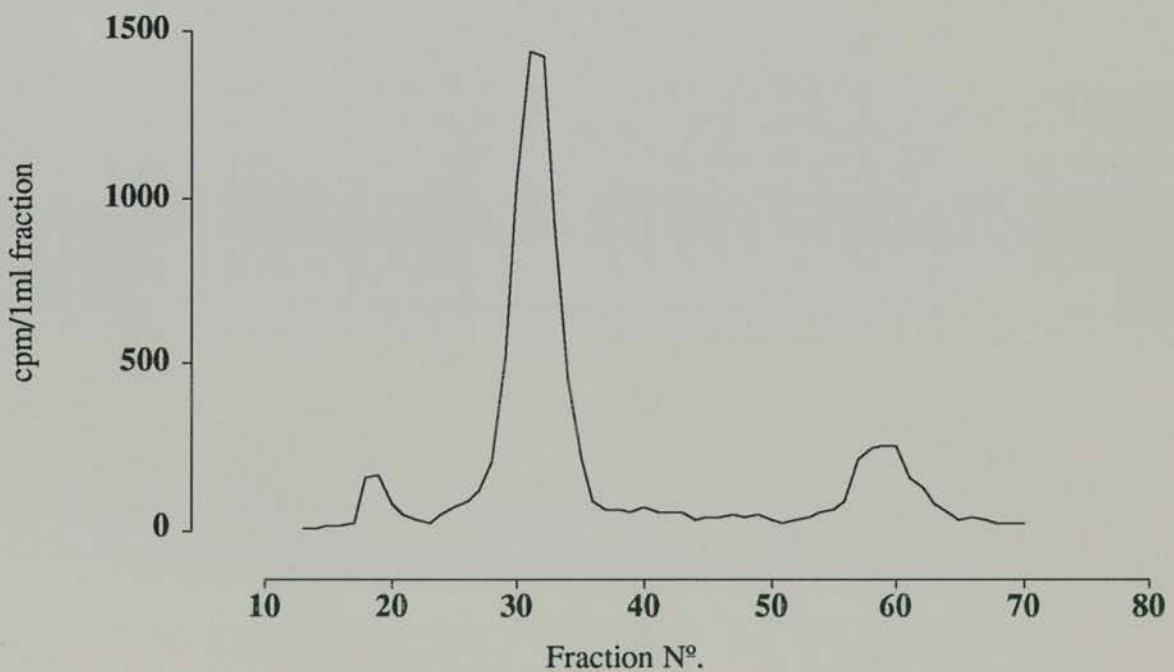

Figure 3.4. Radioactivity eluted in 1ml fractions from a Sephadex G 50-superfine column loaded with [^{125}I]-IGF-I.

was the void volume (V_0) of the column. A range of molecular weight markers were then used to characterise the column in more detail. The trace from the U.V. detector is shown in Figure 3.3. The two larger markers were eluted in the void volume.

The characteristic behaviour of free IGF-I on the column was examined by loading radiolabelled IGF-I on the column, collecting the resulting fractions and measuring the radioactivity of each. There was a major peak of [^{125}I]-IGF-I and a smaller peak of free ^{125}I (Figure 3.4). The total volume (Vt) of the column was calculated from the small peak of free ^{125}I. The column characterisation results are summarised in table 3.2.; this was confirmed in a second experiment. Unbound IGF-I eluted at a volume of 29-39 ml with a peak at 31 ml, within the total column volume. This peak is clearly distinct from the peak of cytochrome-C (12.4 kDa). At present no IGF binding protein has been characterised with a size less than 21.5 kDa. Thus, the column appeared to separate IGF-I from its binding proteins.

Table 3.2 Elution volumes of proteins and radioisotopes on a sephadex G50-fine chromatography column.

MARKER	ELUTION VOLUME (ml)
Blue Dextran (Vo)	18.5
Bovine Serum Albumin (66 kDa)	18.5
Carbonic Anhydrase (29 kDa)	18.5
Cytochrome C (12.4 kDa)	21.5
Aprotinin (6.5 kDa)	34.5
^{125}I (Vt)	57-62 (peak at 59)
[^{125}I]-IGF-I	27-36 (peak at 31)

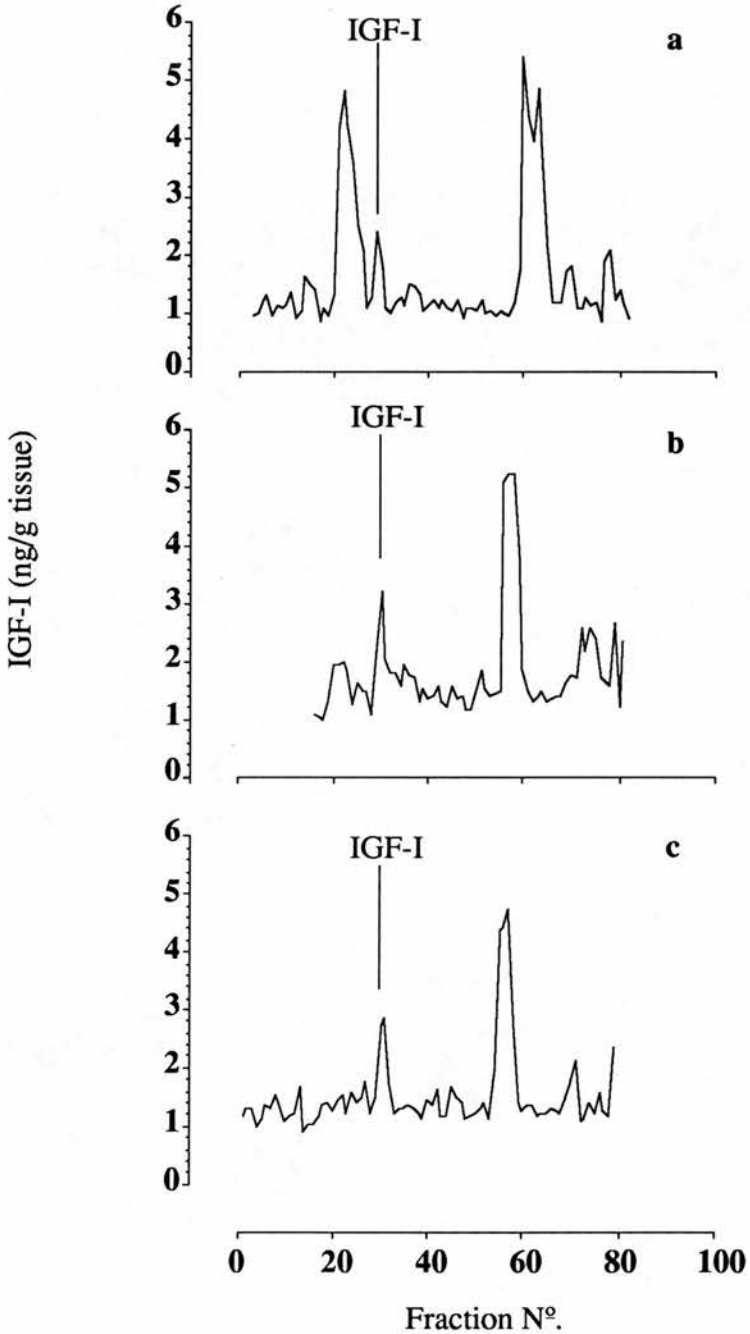

Figure 3.5. IGF-I concentrations in granulosa (**a**), thecal (**b**) and liver (**c**) samples which had been subjected to acid chromatography. Peaks corresponding to immuno-active IGF-I are appropriately labelled. Measurement was by radioimmunoassay, fractions were 1 ml volume.

3.3.2.2. Acid chromatography of samples

Samples of granulosa, thecal and liver tissues (0.5 g of each) were homogenised in acetic acid. The homogenates (0.5 ml of 0.6 g/ml) were loaded on the column and run through it. Fractions of the eluate (1 ml) were collected, then assayed for IGF-I. IGF-I activity was present in all three samples which were analysed (Figure 3.5). In each sample a peak of activity was observed in the range 27-36 ml indicating the presence of free IGF-I. In theca and granulosa samples, activity was also present in the void volume and had therefore eluted with an apparent weight larger than expected. In all three samples activity eluted at Vt implying that degradation of the peptide had occurred.

This experiment shows that the different tissues examined respond differently to acid chromatography, i.e. the separation of IGF-I from binding proteins appears to be dependent on the tissue type.

3.4. Detection Of IGF-I In Granulosa And Thecal Cells *In Vitro*

The expression of IGF-I in cultured granulosa and thecal cells was investigated by immunocytochemical staining. Cells were prepared for culture as described in Section 4.2. After 24 hours culture the cells were washed several times with serum-free medium and then fixed with paraformaldehyde. Cells were incubated with an anti-IGF-I antibody (Armstrong *et al.*, 1990) at dilutions of 1 : 1000 and 1 : 2000, or with control normal rabbit serum (NRS). In order to determine the non-specific nature of the antibody an aliquot of the antiserum was negated by pre-incubation with IGF-I and then used as described. Following staining with DAB the cells were photographed.

Both the thecal and granulosa cells were stained when incubated with the anti-IGF-I antibody but not when incubated with non-immune rabbit serum. Staining was negated by pre-incubation of the antiserum with IGF-I. The staining of both cell types was less intense when the antiserum was used at a dilution of 1:2000 than at a dilution of 1:1000.

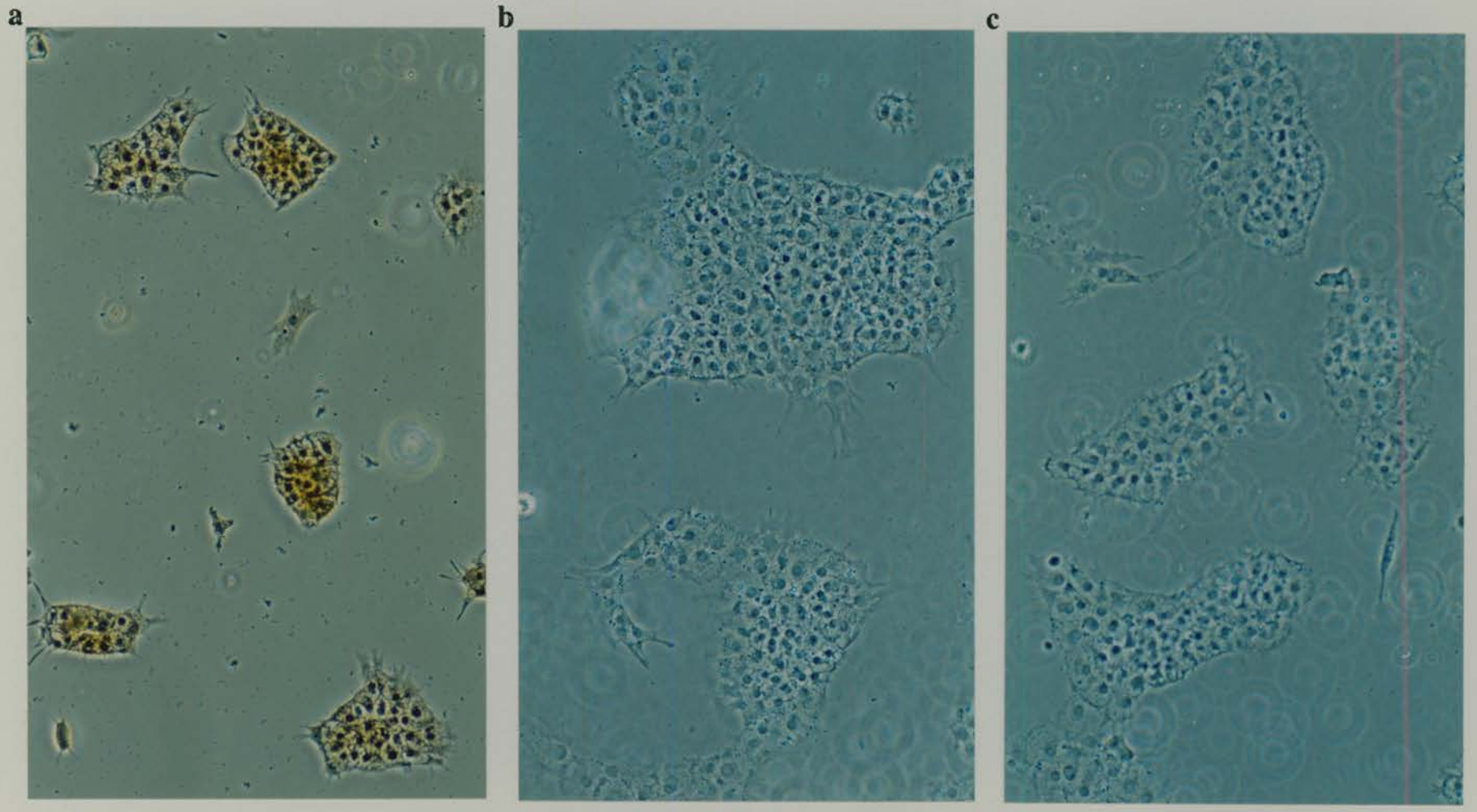

Figure 3.6 Cultured granulosa cells treated with (a) anti-IGF-I antiserum at adilution of 1:1000, (b) with anti-IGF-I antiserum negated with 5µg of IGF-I and (c) with the control normal rabbit serum. The cells were further subjected to immunocytochemical staining with DAB.

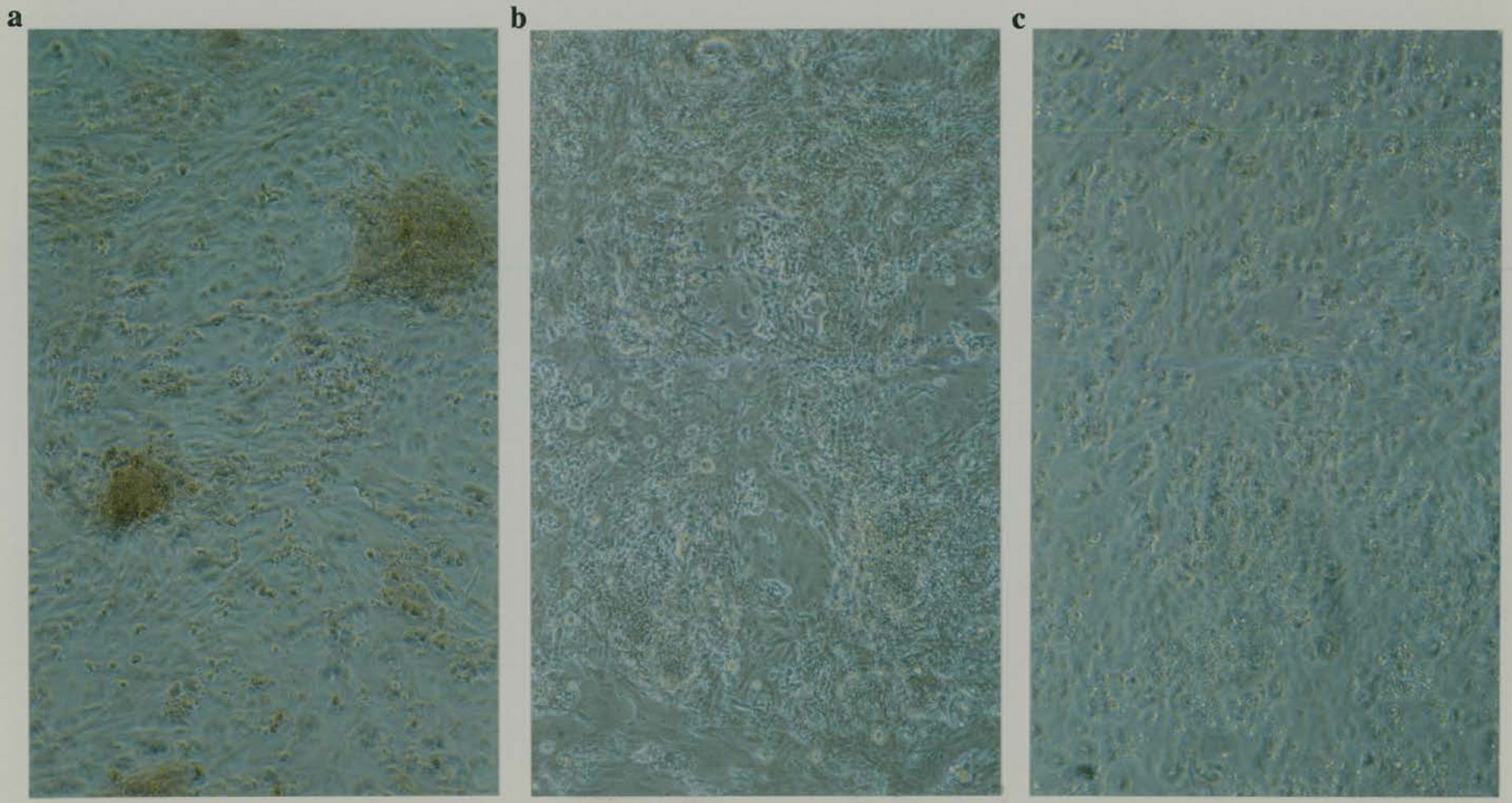

Figure 3.7 Cultured thecal cells treated with (a) anti-IGF-I antiserum at adilution of 1:1000, (b) with anti-IGF-I antiserum negated with 5µg of IGF-I and (c) with the control normal rabbit serum. The cells were further subjected to immunocytochemical staining with DAB.

Cells containing immunocytochemically identified IGF-I are shown in Figures 3.6. and 3.7. Granulosa cells incubated with the anti-IGF-I antibody at 1 : 1000 with and without negation with 5 μg IGF-I or incubated with the control non-immune rabbit serum are shown in Figure 3.6. Thecal cells subjected to the same treatments are shown in Figure 3.7. These results indicate that R2/2 specifically stained IGF-I in both thecal and granulosa cells, showing that the peptide is present in both cell types *in vitro*.

3.5. SUMMARY AND DISCUSSION

Granulosa and thecal tissues were shown to express IGF-I mRNA in experiments which used reverse transcription of extracted RNA and specific amplification of IGF-I cDNA produced by the polymerase chain reaction (RT-PCR). Further experiments in which RNA was extracted from follicles at different stages of development showed that both tissues expressed IGF-I mRNA at all the stages examined (F1, F2, F3 and F4 follicles). IGF-I mRNA expression in chickens is therefore different from that observed in the rat since rat thecal cells do not express this gene (Oliver *et al.*, 1989) . The chicken ovary also differs from the human ovary, since IGF-I gene expression was not detected in human granulosa cells (Geisthovel *et al.*, 1989). Thus no general pattern of ovarian IGF-I gene expression has emerged from the studies in mammals or birds as yet.

The PCR amplifies the original transcribed DNA by up to 10^9-fold in a 30 cycle reaction. One consequence of this is that differences in the efficiency of the reverse transcription reactions and errors in aliquoting the RNA starting material result in large differences in the amount of PCR product. For this reason the results could not be used to quantify differences in IGF-I mRNA expression. This was emphasised by the study described in Section 3.2.4. in which the first experiment indicated possible developmentally-related changes in IGF-I gene expression in thecal tissues (Figure 3.2), but when this was repeated no such change was evident. Further work is therefore required to determine whether there are developmentally, or tissue-related changes in IGF-I expression in chicken follicles.

The concentrations of IGF-I itself was measured in extracts from granulosa and thecal tissues. Initially a method developed by D'Ercole *et al.* (1984) and modified by Enright *et al.*(1989) was used. This showed that IGF-I was present in both the tissues, in concentrations greater than in liver. This suggests that both tissues in the pre-ovulatory follicle which were examined, are important sites of production and possibly action.

The effects of the binding proteins on immunoassays are complex and difficult to predict since binding proteins may compete with anti-IGF-I antibodies for binding of [^{125}I]-IGF-I, this could result in higher estimates of concentrations of IGF-I than those actually present. However, this effect is counterbalanced by an underestimate of IGF-I due to binding of unlabelled IGF-I by binding proteins (Daughaday *et al.*, 1987). Methods to separate IGF-I from their BPs by acidification, followed by precipitation with ethanol (Daughaday *et al.*, 1980) have been criticised for incomplete removal of BPs in more recent studies (Daughaday *et al.*, 1987; Holland *et al.*, 1988; Mesiano *et al.*, 1988). Therefore, to remove the interfering effects of binding proteins and thus measure IGF-I tissue concentrations more accurately than by extraction alone, acid extracted samples were subjected to acid chromatography. However, significant IGF-I immunoactivity was present with larger eluted proteins in both granulosa and thecal samples, possibly IGF-I binding proteins. This indicated that the separation of peptide from binding protein was not complete in this experiment. Interestingly, the relative order of concentrations of the protein fraction associated with IGF-I is the same as that of the peptide as measured without chromatography. A possible explanation of this is that IGFBP concentrations vary between the three tissues rather than the free peptide, and it was these that were measured in the extracted samples and not the free peptide. This was not proved in experiments described here, further work with a cIGFBP RIA may elucidate this situation.

The third part of the experimental study described in this chapter confirmed, immunocytochemically, that the peptide was present in both granulosa and thecal cells *in vitro*.

CHAPTER 4: THE EFFECTS OF IGF-I AND GONADOTROPHINS ON OVARIAN CELLS *IN VITRO*

4.1. INTRODUCTION

Tissue culture systems for chicken granulosa and thecal cells were developed to examine the effects of exogenous IGF-I and its interactions with gonadotrophins on the DNA synthesis of these cells. Cell cultures derived from follicles at different developmental stages were used to determine whether the effects of IGF-I or gonadotrophins on DNA synthesis are developmentally-regulated.

4.2. CELL CULTURE SYSTEM DEVELOPMENT

Existing tissue culture methods were adapted to establish a system for maintaining both granulosa and thecal cells in culture. The conditions considered in this exercise were the dispersal methods, attachment conditions and cell plating density. Granulosa and thecal tissues are quite different in their structure; granulosa cells are epithelial-type cells arranged in a single-cell layer, while the theca is more fibrous comprising a mixed population of cells, the majority of which are fibroblasts. The cells of the granulosa layer are consequently easier to disperse than those of the thecal layer. For these reasons different approaches were taken to the development of culture systems for these cells.

4.2.1. Granulosa cell culture system

The method of dispersion was esablished by adapting the protocol published by Zakar and Hertelendy (1980a); this protocol involves enzymatic dispersion by incubation of the granulosa layer for 10 minutes at 37°C with a collagenase solution followed by mechanical dispersion by continuous pipetting of the cell suspension. The 10 minute incubation was found to be unecessary for good dispersal of the cells, therefore the method was simplified to a single step in which cells were suspended in a collagenase solution in M199 (5mg collagenase per ml) at room temperature and

pipetted continuously for approximately 1-3 minutes. Following dispersal the collagenase was removed by washing the cells in M199 as described in Chapter 2. Cell viability of dispersed cells was measured by the trypan blue exclusion test, also described in Chapter 2, which showed it to be invariably greater than 95%.

Experiments were conducted to optimise the attachment of granulosa cells to plates. Tissue culture plates referred to as "untreated" in these experiments had, however, been treated during manufacture by corona discharge and radiation. Both these processes enhance the attachment of cells to the plastic surfaces (see Section 2.4.5.).

In an initial study granulosa cells suspended in M199 (at 5 $x10^5$ cells per ml) with or without 10% (v/v) fetal calf serum (FCS) were seeded on "untreated" plastic multiwell tissue culture plates. The unsupplemented cells did not attach to the wells; however cells suspended with FCS did attach, but the efficiency estimated by microscopic examination was very low. Several factors are known to affect the attachment of many types of dispersed cells to plastic surfaces. Therefore, comparisons of granulosa cell attachment to: "untreated" plates (group 1); plates pre-incubated for 24 hours with neat FCS or donor bovine serum (DBS) then aspirated (group 2); plates pre-coated with gelatin, fibronectin, or collagen (group 3) were made. The cells were seeded in suspensions containing 5 or 10% FCS (v/v) and incubated at 41°C and 5% CO_2.

Examination of the plates after 24 hours revealed that cell attachment had occured to the surfaces in all the treatment groups. However, the degree of attachment was greatest on the plates of group 3. After 72 hours of culture no cells remained attached on the plates of group 1 and there was a marked difference in the degree of attachment to the plates of groups 2 and 3. After 120 hours the cells on the plates in group 3 remained attached and appeared healthy. Thus pre-treating the plate surface affected the efficiency of granulosa cell attachment.

To determine the optimum plate-coating material, granulosa cells were seeded on plates coated with the treatments listed in group 3 above. Following 120 hours of culture, the medium was changed to M199 containing 0, 1, 3 or 5% FCS (v/v). The

incorporation of [^{3}H]-thymidine into these cells following 24 hours of these treatments was then measured using the method described in Chapter 2. The results (Figure 4.1) were analysed by Student's t test (unpaired, two-tailed) which showed that there were no significant differences in the DNA synthesis response to FCS between cells on plates coated in three different ways. The results also showed that the response was significantly different between zero and 3% FCS treated cells on all plates, the differences with the zero treatment were only significant for cells on fibronectin-coated plates at 1% FCS and only cells on collagen-coated plates at 5% FCS stimulation.

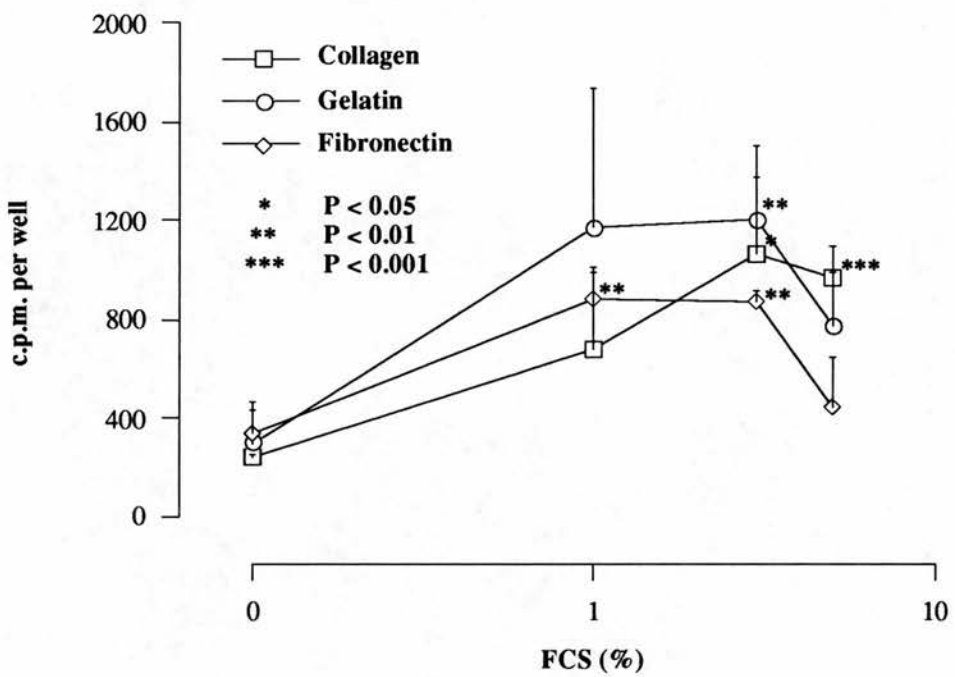

Figure 4.1 Mean incorporation of [^{3}H]-thymidine by triplicate cultures of granulosa cells seeded on plates coated as indicated and untreated or treated by fetal calf serum (FCS). Standard deviation of the mean is shown as a vertical line. Significant differences (P values) of incorporation by treated cells compared with untreated cells are indicated.

These results led to the use of gelatin for pre-coating of plates in the granulosa cell culture system, since gelatin is the most economic of the three treatments, and the inclusion of 3% FCS in medium to facilitate the attachment of the cells.

In order to determine the optimum cell density for plating granulosa cells in this system, cells were dispersed and suspended in medium containing 3% FCS at densities ranging from 2 $x10^4$ to 1.5 $x10^6$ cells / ml of medium. These suspensions were then plated on 48-well plates which had been gelatin treated at 0.5ml per well (area of each well = $1cm^2$), six wells were plated per density group. Following the cell attachment period (48 hours) the medium in the cultures was changed with fresh medium containing 0.01% FCS (v/v) for 24 hours, whereupon this was repeated and half the cultures were treated with 3% FCS (v/v). The incorporation of [^{3}H]-thymidine into the cells following 24 hours of treatment was measured as described in Chapter 2.

The total radioactivity from each triplicate set of cultures was measured and the mean and standard deviation calculated. These values were plotted against density and the resulting graph is shown in Figure 4.2. Uptake of [^{3}H]-thymidine by granulosa cells treated with serum was greatest when they were seeded at a density of 5 $x10^4$ cells per $1cm^2$ well.

4.2.2. Thecal cell culture system

Methods published by Marrone and Hertelendy (1983) and Tilly and Johnson (1989) were adapted to establish a protocol for the dispersion and purification of chicken thecal cells. A different approach from that used to culture granulosa cells was necessary since the thecal layer is more fibrous and contains blood vessels. This requires more digestion to disperse the cells and the removal of contaminating red blood cells (RBC). The main features of this method, (described in detail in Section 2.2.4.2.) are enzymatic digestion in collagenase solution for 90 minutes at 37°C with regular pipetting of the suspension and the removal of red

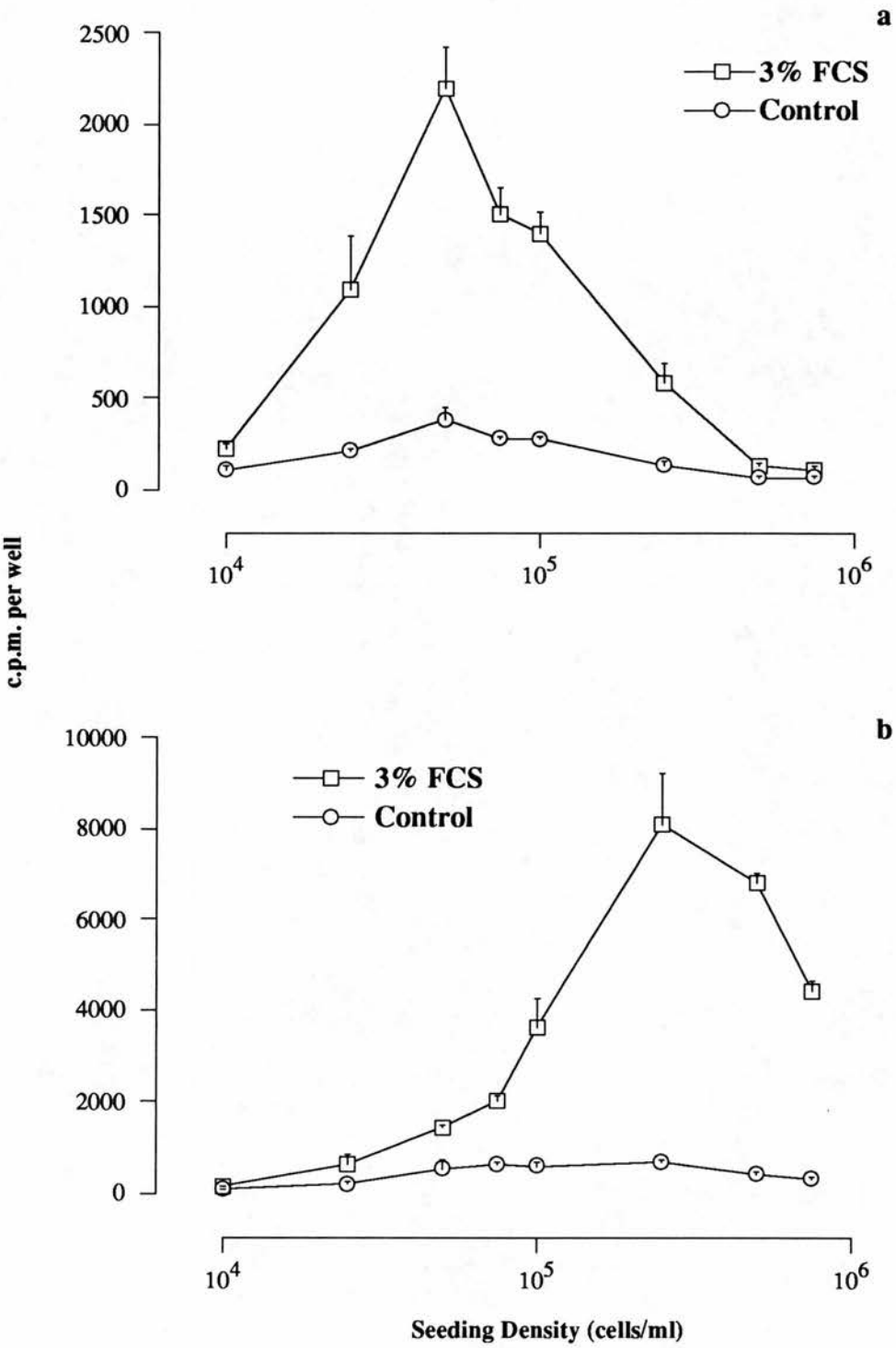

Figure 4.2 Mean uptake of [^{3}H]-thymidine by granulosa (**a**) and thecal cells (**b**), cultured in triplicate and seeded at different densities. Cells were untreated (control) or treated with fetal calf serum (3% FCS).

blood cells on a percoll gradient followed by lysis. Following these procedures, cells were washed as in the method for preparing granulosa cells. The viability of dispersed thecal cells was routinely measured by trypan blue exclusion and found to be 70-80%.

Initial experiments were conducted to examine the plating efficiency of dispersed thecal cells. Cells were seeded on plastic mutiwell tissue culture plates which were untreated or had been treated with collagen or gelatin. Examination of cultures after 24, 48 and 72 hours showed that the plating efficiency was good in all three groups and that there was no visible difference between the groups. The supplementation of the medium with 3% FCS was found to enhance the plating efficiency compared with unsupplemented controls. Therefore in further experiments thecal cells were cultured on untreated plastic plates in medium supplemented with 3% FCS.

In order to determine the optimum plating density, an experiment was conducted with thecal cells, identical in design to that described for granulosa cells in Section 4.2.1. The results shown in Figure 4.2 indicate that [^{3}H]-thymidine incorporation by thecal cells stimulated with serum was greatest in cells cultured at a density of 2.5×10^5 cells per 1cm^2 well.

4.2.3. The granulosa and thecal cell culture systems

Tissue culture systems were based on the preliminary experiments described above in Sections 4.2.1 and 4.2.2. These systems include a growth restriction step, during which the cells are cultured in medium with a very low serum content (0.01% v/v) for 24 hours. This step is to make cells enter the resting stage (G0) of the cell cycle. This results in synchronisation of their DNA replication cycles when stimulated with growth factors. The main features of these systems are shown in Table 4.1. below.

Table 4.1. Culture system conditions for chicken granulosa and thecal cells.

Conditions	Granulosa Cells	Thecal Cells
Incubation Temperature	41°C	41°C
CO_2	3.5 %	3.5 %
Treatment of culture dishes	Gelatin	None
Serum in medium for plating	3%-5%	3%-5%
Plating density	$5x10^4$ cells/well	$2.5x10^5$ cells/well
Cell attachment period	48 hours	48 hours
Restriction period	24 hours	24 hours

4.2.4. Morphology of granulosa and thecal cells *in vitro*

During the preliminary experiments described in 4.2.1 and 4.2.2, both cell types were photographed. Figure 4.3 shows photomicrographs of granulosa and thecal cells 72 hours following initial seeding of the cells on plastic, multi-well plates. Granulosa cells which are initially round following dispersal become flattened on attaching to the plates. Thecal cell cultures are heterogeneous; two cell types were clearly visible, these were elongated fibroblasts and round cells, presumed to be steroidogenic cells. The round cells were aggregated into well separated clumps on the plate, the intervening areas of the plate were covered by the fibroblasts.

4.3. The Effects Of Serum On Follicular Cells *In Vitro*

The effect of serum on DNA synthesis was investigated in granulosa and thecal cells prepared from the three largest pre-ovulatory follicles of a single hen (F1, F2 and F3), pooled and cultured as described in Section 2.4.5. The cells were treated with FCS at concentrations from 0.1% to 10% (v/v) or with medium

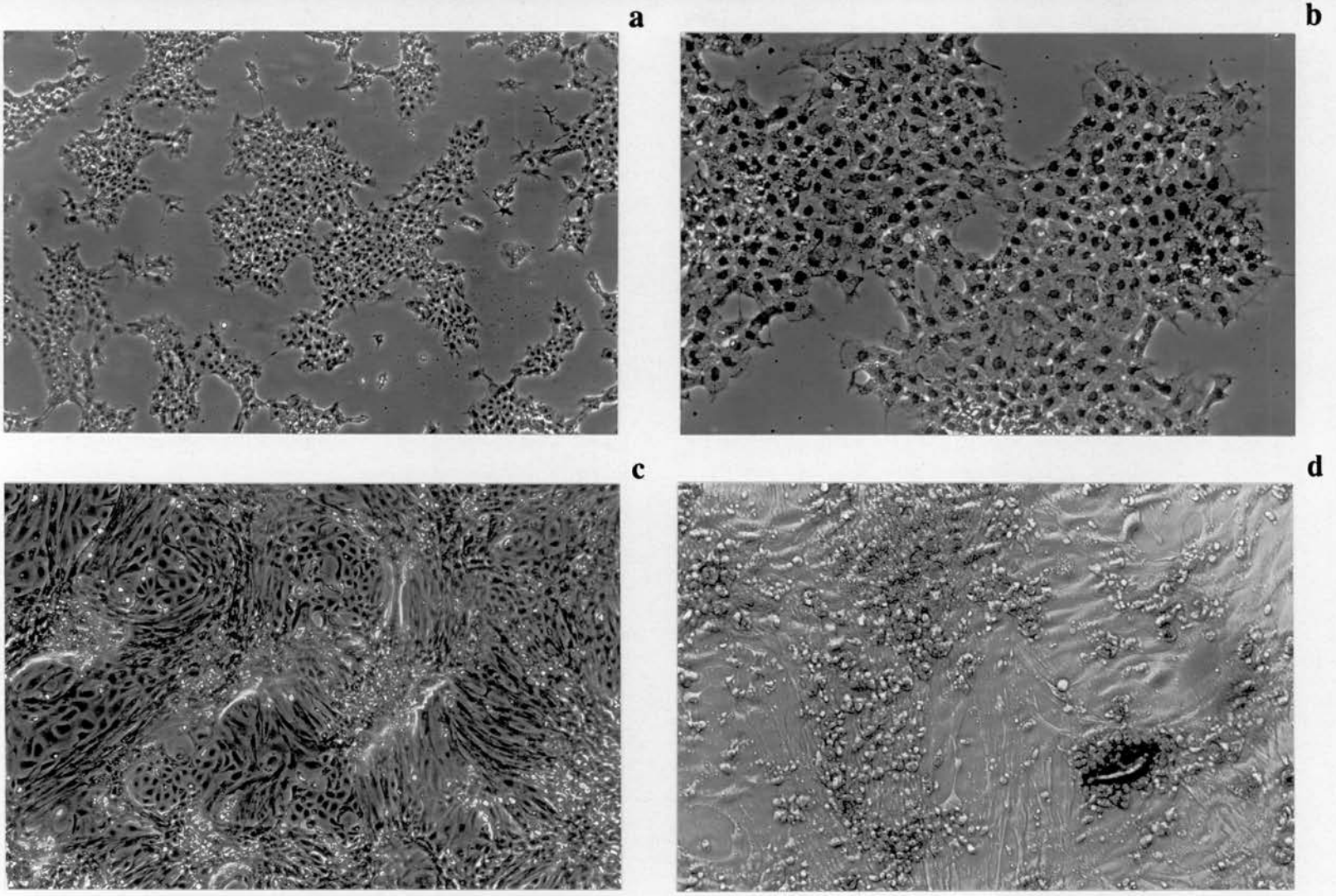

Figure 4.3 Photomicrographs of granulosa cells at 100 x (a) and 200 x (b) magnifications and thecal cells at 100 x (c) and 200 x (d) magnifications. Cells of both types were photographed following 72 hours of culture in conditions described in Table 4.1.

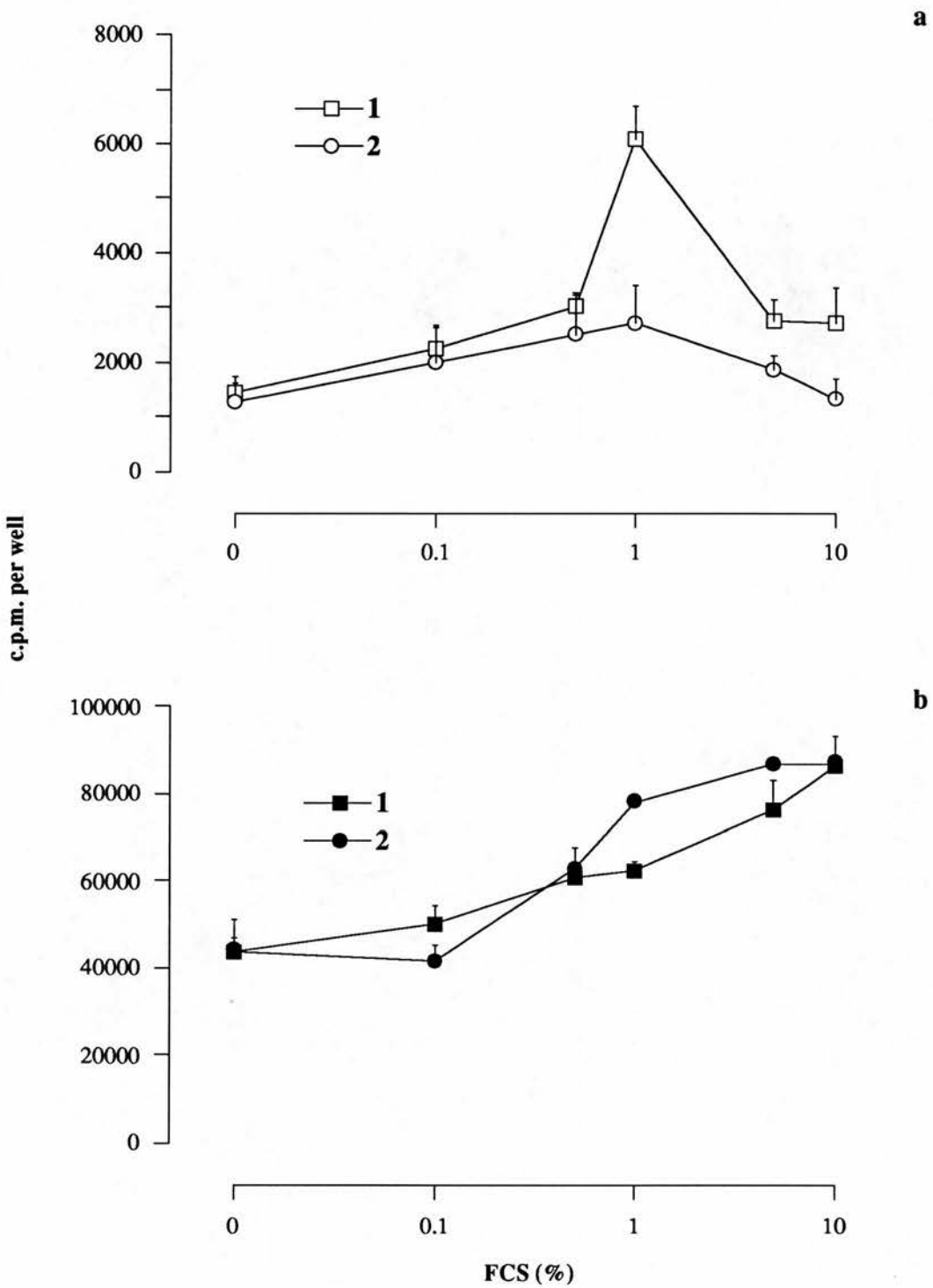

Figure 4.4. The mean incorporation of [^{3}H]-thymidine by granulosa (**a**) and thecal cells (**b**), cultured in triplicate and treated with fetal calf serum (FCS) in two independent experiments (1 and 2).

containing no serum. The effects of this treatment on [^{3}H]-thymidine incorporation into the cultured cells are shown in Figure 4.4.

Both granulosa and thecal cells responded to FCS by incorporating [^{3}H]-thymidine in a dose-dependent manner, the ED_{50} values of these responses were 0.7 and 0.3 % FCS for granulosa and 1 and 0.5 % FCS for thecal cells. For granulosa cells there was a maximum response between the 1% and 5% FCS. The maximum response of thecal cells to serum stimulation did not appear to have been achieved in the range of concentrations used.

The greatest responses of thecal cells to stimulation were 14.2 and 14.4-fold greater than the greatest response of granulosa cells in experiments 1 and 2 respectively. When calculated as the response per plated cell, the maximum responses of thecal cells were 2.8 and 2.9-fold greater than those of granulosa cells in each experiment.

4.4. The Effects Of IGF-I And Gonadotrophins On Ovarian Cells *In Vitro*

The mitogenic effects of IGF-I and gonadotrophins separately or in combination on granulosa and thecal cells were determined *in vitro*. In addition, the steroidogenic responses to gonadotrophin treatments were determined to confirm the biological activity of these hormone preparations.

Ovine gonadotrophins were used because purified chicken (c) LH was in very short supply and pure cFSH was unavailable. However, a limited quantity of cLH was available and was used to repeat some of the experiments involving oLH in order to determine whether there is any difference in the action of the two LHs in the *in vitro* system.

In the experiments described below, the cells were obtained from the three largest pre-ovulatory follicles of hens (F1, F2 and F3) and placed in granulosa or thecal cell pools.

4.4.1. The mitogenic response of ovarian cells to IGF-I *in vitro*

Granulosa and thecal cells were prepared for culture as described in Chapter 2. Cells were seeded and cultured according to the protocol described in Table 4.1. The response of both cell types to IGF-I was examined by adding different doses of the peptide and then measuring the stimulatory effect on the incorporation of [^{3}H]-thymidine into the DNA of the cells.

IGF-I doses ranged from 0.5 to 100 ng/ml. In addition, cells were stimulated with 3% (v/v) heat inactivated FCS as a positive control for the stimulation of [^{3}H]-thymidine incorporation in both granulosa and thecal cells. As a negative control, cells were cultured during the treatment period in medium with a very low FCS content (0.01% v/v). The treatments were carried out on triplicate cultures. In each experiment the granulosa and thecal tissues were obtained from the same hen, the experiments were repeated twice.

IGF-I stimulated uptake of [^{3}H]-thymidine in a dose-dependent manner by both granulosa and thecal cells (Figures 4.5 and 4.6). All the cultures responded to FCS treatment (positive control) with an increased [^{3}H]-thymidine incorporation compared with untreated cells. The ED_{50} values (± S.E.M.) of IGF-I were 7.37 (± 1.46), 11.61 (± 7.28) and 11.83 (± 2.57) ng/ml for triplicate granulosa cell cultures prepared from the three individual birds and 20.05 (± 28.31), 15.16 (± 4.70) and 7.05 (± 0.60) for triplicate thecal cell cultures.

The difference in the uptake of [^{3}H]-thymidine by serum-treated cultures, compared with that in the untreated control cultures was measured for granulosa and thecal cells. The value for thecal cultures was significantly greater per plated cell than that in granulosa cell cultures, $P < 0.01$ (Figure 4.7).

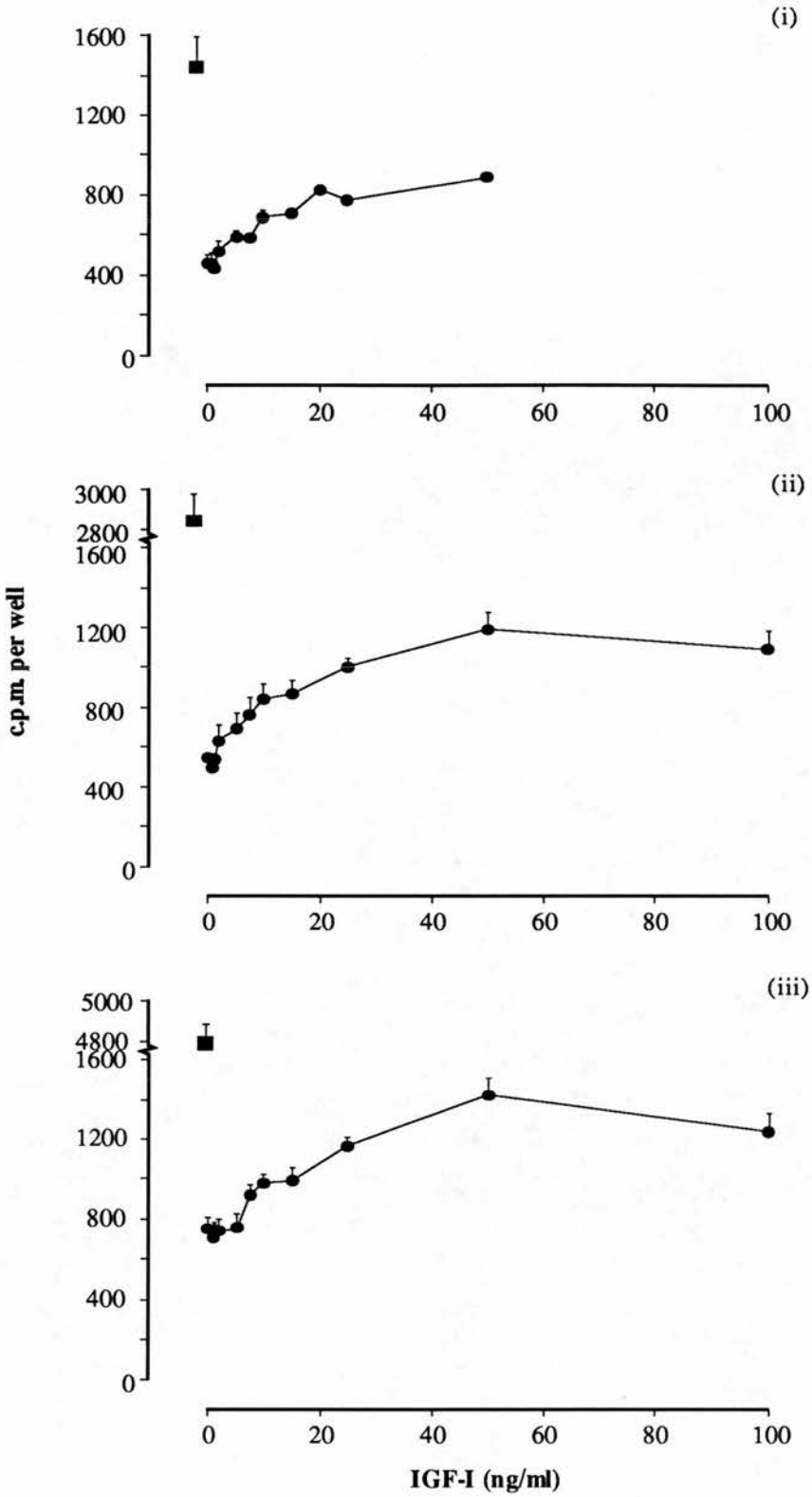

Figure 4.5. Mean incorporation of [^{3}H]-thymidine by granulosa cells cultured in triplicate and treated with IGF-I (●) in three separate experiments (i, ii and iii). The incorporation in the presence of 3% fetal calf serum is also shown (■). The standard deviation of the mean is shown as vertical lines.

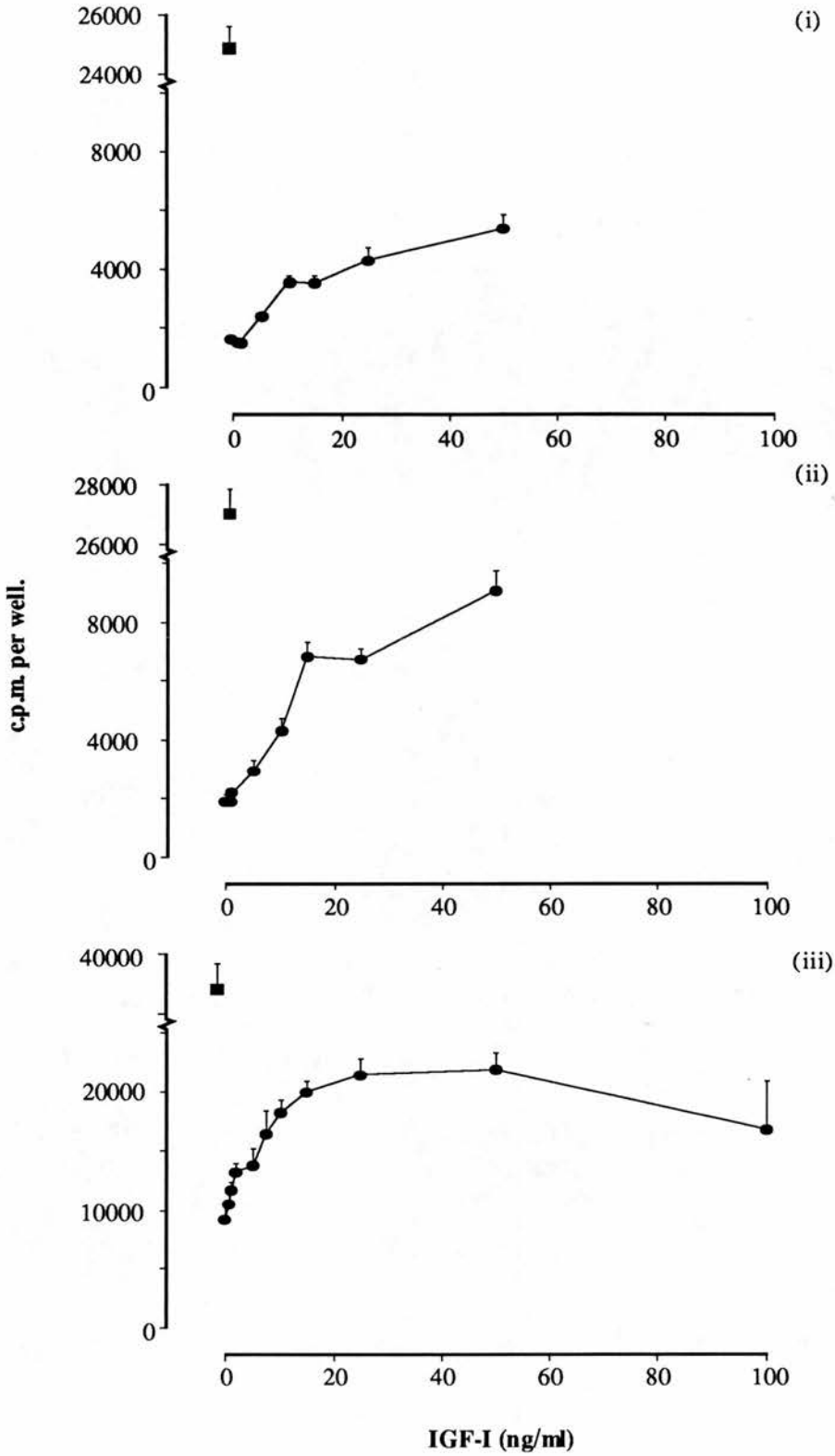

Figure 4.6. Mean incorporation of [^{3}H]-thymidine by thecal cells cultured in triplicate and treated with IGF-I (●) in three separate experiments (i, ii and iii). The incorporation in the presence of 3% fetal calf serum is also shown (■). The standard deviation of the mean is shown as vertical lines.

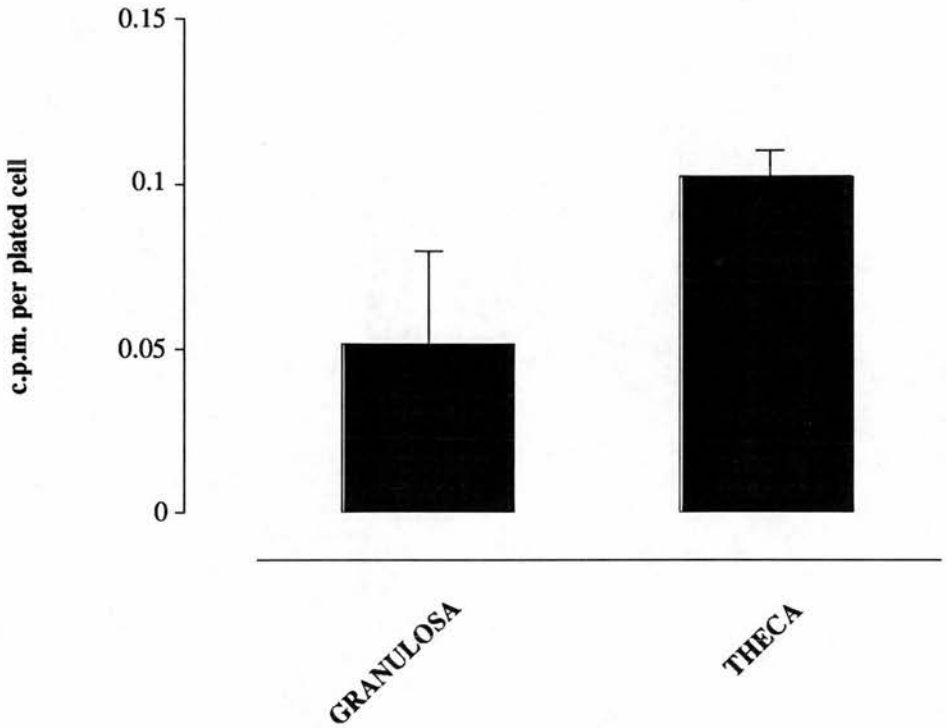

Figure 4.7 Differences in the mean incorporation of [^{3}H]-thymidine into triplicate untreated cell cultures and into those cultured in the presence of 3% fetal calf serum. The incorporation is expressed as c.p.m. per cell plated.

4.4.2. The steroidogenic response of granulosa and thecal cells *in vitro*

Measurement of the production of progesterone by granulosa cells and the production of androstenedione by thecal cells treated with LH and FSH , respectively, was used to assess the steroidogenic response of these cells. Granulosa cells were stimulated with ovine (o) LH or chicken (c) LH for 24 hours; thecal cells were stimulated with oFSH. The medium conditioned by the granulosa and thecal cells during the treatment period was aspirated and analysed by radioimmunoassay for progesterone and androstenedione content respectively.

Granulosa cells responded to both oLH and cLH by producing progesterone in a dose-dependent manner (Figure 4.8). The ED_{50} value for oLH (± S.E.M.) was 19.82 ng/ml (± 4.14) (Figure 4.8 a) and for cLH, 26.3 ng/ml (± 6.29) (Figure 4.8 b). There was no significant difference in the response of granulosa cells to the two LHs. In two independent experiments, thecal cells responded to oFSH by

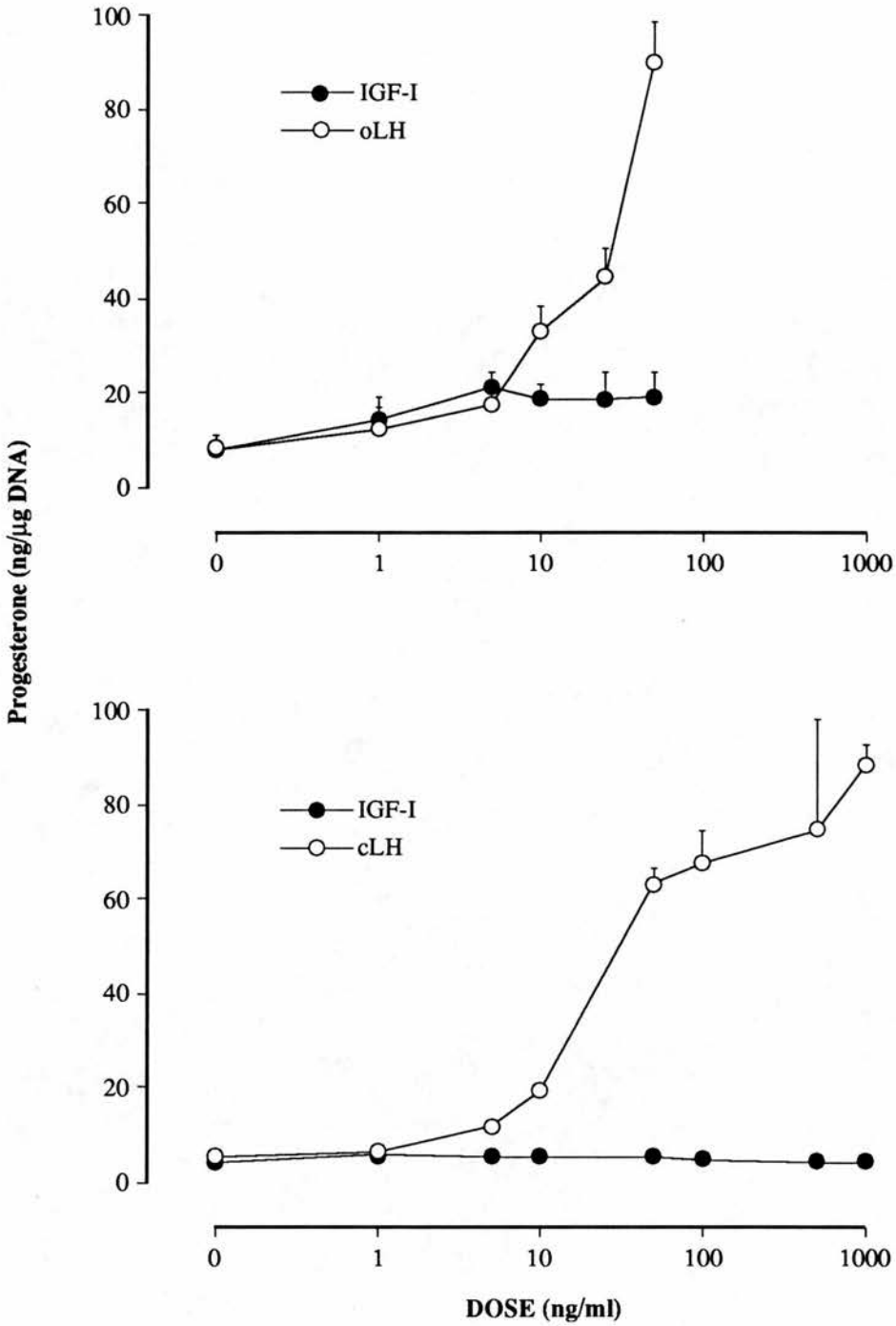

Figure 4.10. Progesterone production by granulosa cells treated with oLH or IGF-I in two separate experiments, measured by radioimmunoassay. The means and standard deviations (vertical bars) of triplicate cultures are shown, The DNA content of the cultured cells was measured following treatment and used to express the progesterone concentration.

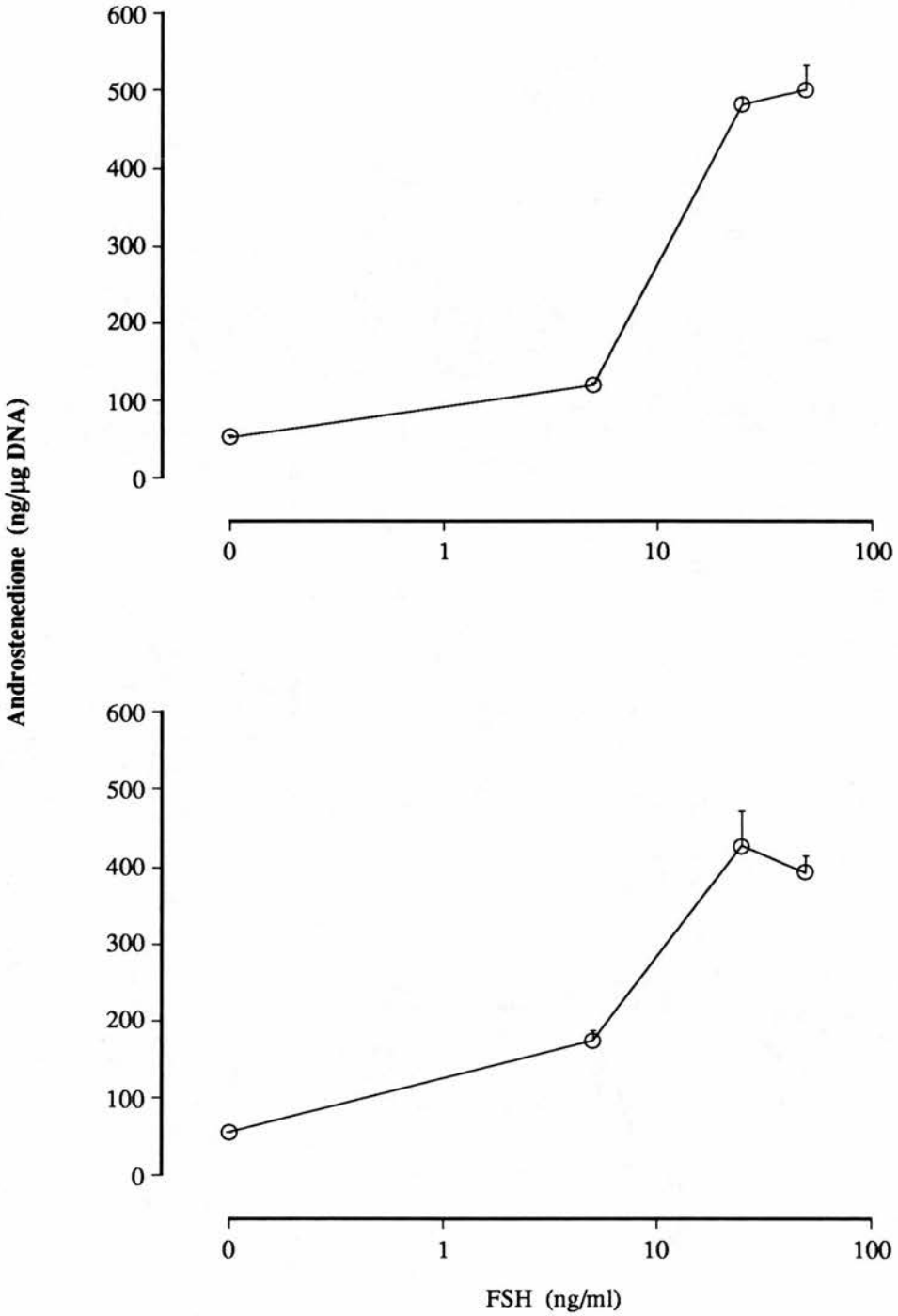

Figure 4.9. Androstenedione production by cultured chicken thecal cells treated with ovine (o) FSH in two separate experiments, measured by radioimmunoassay. The means and standard deviations (vertical bars) of triplicate cultures are shown. The DNA content of the cultured cells was measured after stimulation and used to express the androstenedione concentration.

producing androstenedione (Figure 4.9). The ED_{50} values for oFSH (± S.E.M.) were 8.8 ng/ml (± 0.1) (Figure 4.9 a) and 6.5 ng/ml (± 1.6) (Figure 4.9 b) in the two independent experiments.

4.4.3. The mitogenic effects of gonadotrophins on granulosa and thecal cells *in vitro*

Granulosa and thecal cells were treated with oLH or oFSH in order to determine their possible mitogenic effects on cells in the culture system. These effects were examined by measuring the resulting stimulation of thymidine incorporation by the cells.

Cell cultures were treated with a range of doses of LH or FSH up to 50 ng/ml, they were also treated with IGF-I for comparison with the experiments described in Section 4.4.1. Positive and negative control treatments as described above were included. The experiment was repeated with cells prepared from different birds.

Treatment with FSH did not stimulate [^{3}H]-thymidine incorporation above the baseline level of the untreated control in either cell type (Figures 4.10, 4.11). LH treatment caused a dose-dependent increase in thymidine incorporation in granulosa cells in both experiments with ED_{50} values (± S.E.M.) of 14.32 ± 2.57 and 10 ± 1.24, respectively (Figure 4.10), but did not have this effect on thecal cells (Figure 4.11). The granulosa cells responded to IGF-I in a similar (dose-dependent) manner to those in Section 4.4.1, with ED_{50} values (± S.E.M.) of 13.57 ± 1.60 and 10.55 ± 6.01, respectively (Figure 4.10). There was good agreement between the results from the two independent experiments. The ED_{50} values of IGF-I and LH were not significantly different from each other in either experiment

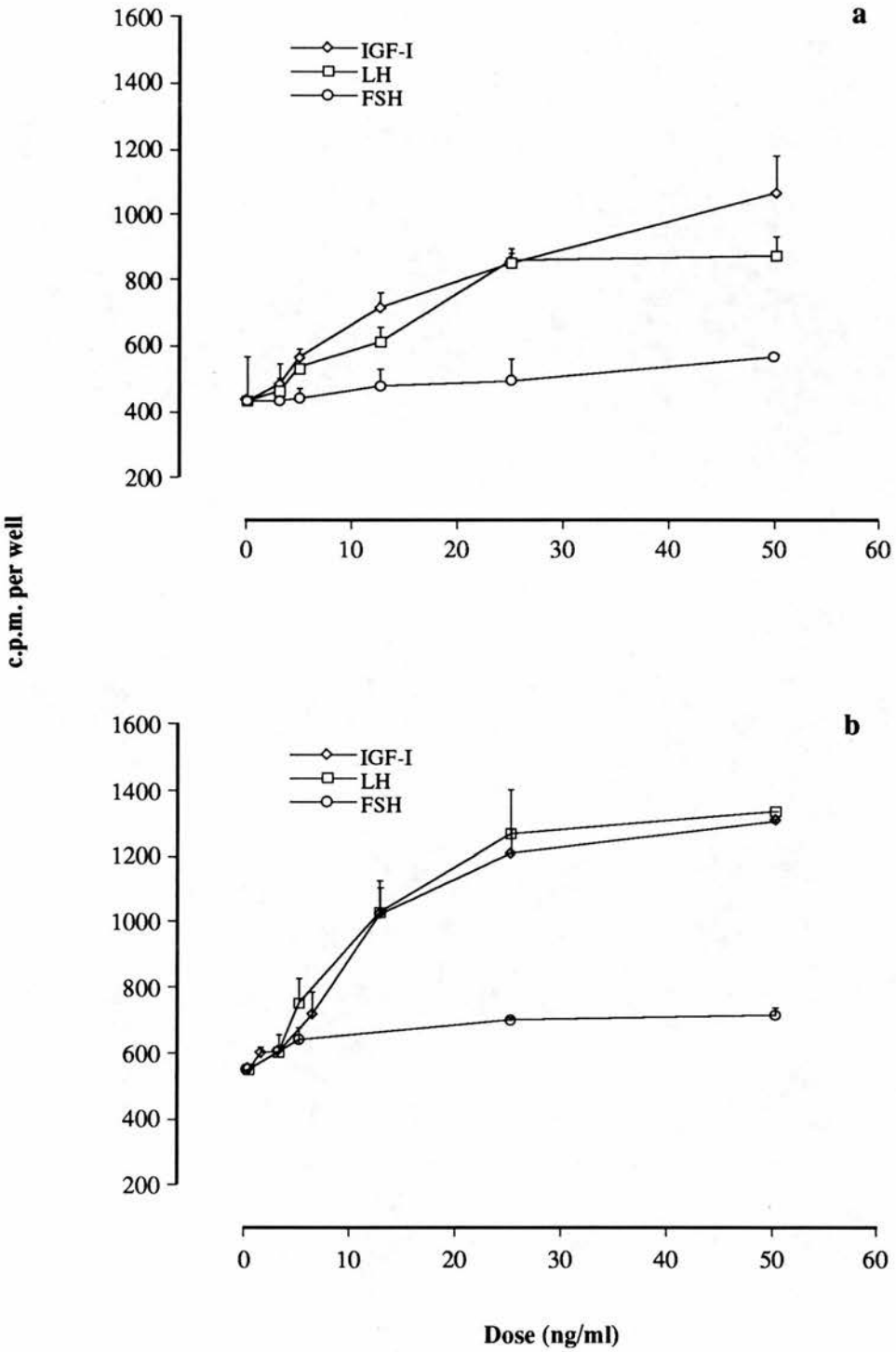

Figure 4.10. Uptake of [^{3}H]-thymidine by granulosa cells in two separate experiments (a and b). Cells were untreated or treated with IGF-I, oLH or oFSH as indicated. Each point represents the mean of triplicate measurements, the standard deviation of the mean is shown as a vertical line around each point.

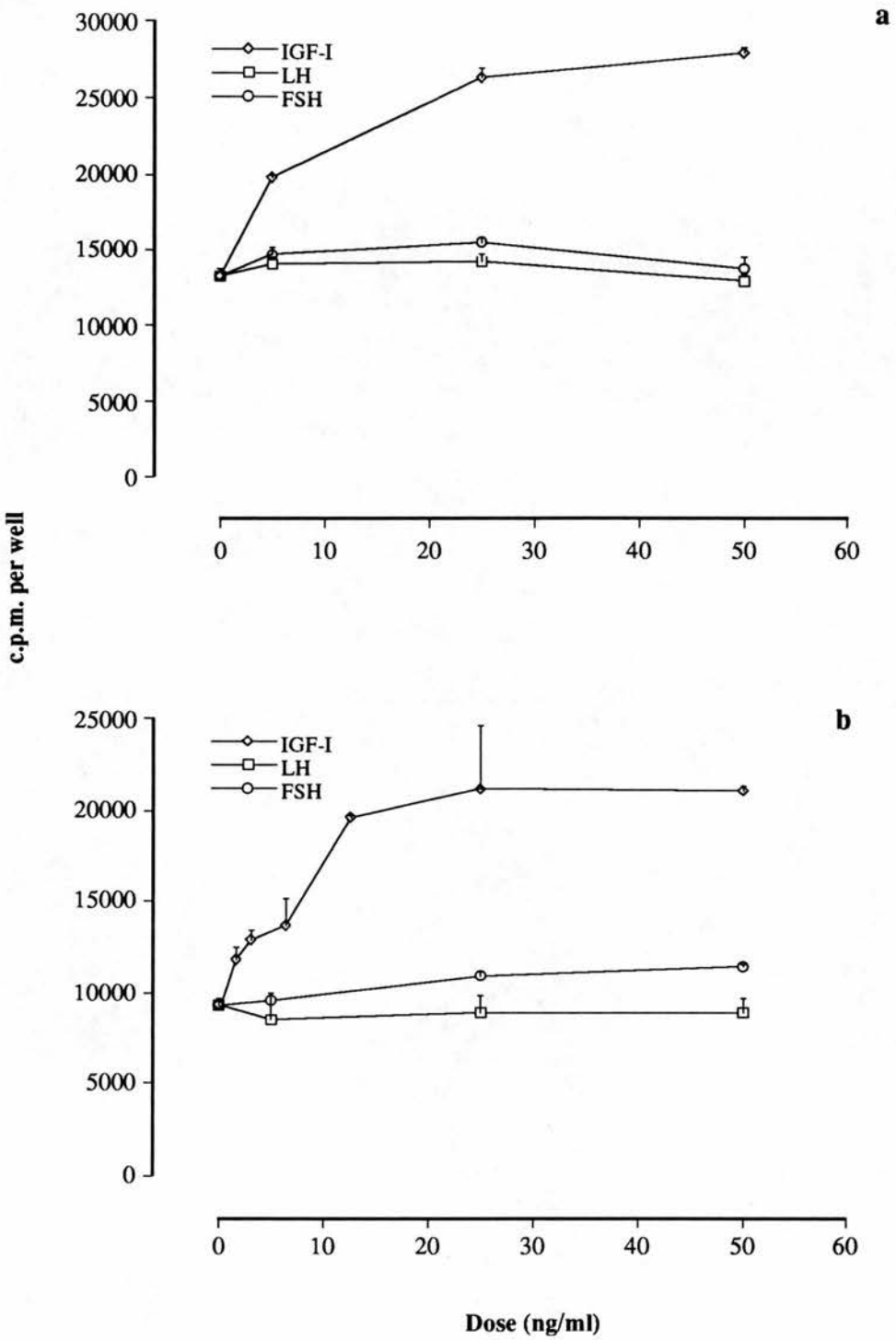

Figure 4.11 Uptake of [^{3}H]-thymidine by thecal cells in two separate experiments (a and b). Cells were untreated or treated with IGF-I, oLH or oFSH as indicated. Each point represents the mean of triplicate measurements, the standard deviation of the mean is shown as a vertical line around each point.

4.5. SYNERGISTIC EFFECTS OF IGF-I AND GONADOTROPHINS ON GROWTH AND STEROIDOGENESIS IN OVARIAN CELLS *IN VITRO*

Work published on the effects of IGF-I in the mammalian ovary, described in Chapter 1, shows that IGF-I and FSH have synergistic effects on steroidogenesis and differentiation of granulosa cells. The possible synergistic effects of IGF-I and gonadotrophins on mitogenesis and steroidogenesis in chicken granulosa and thecal cells were examined in a series of experiments described below.

4.5.1. Uptake of [^{3}H]-thymidine after treatment with IGF-I and mammalian LH and FSH

Cultured granulosa and thecal cells were treated with 25 ng/ml doses of either IGF-I, LH, FSH or IGF-I with LH or FSH. The effects of these treatments on the incorporation of [^{3}H]-thymidine were measured. All treatments were carried out on triplicate wells; the experiment was replicated and the results are shown in Figures 4.12 and 4.13.

No significant difference was found in the incorporation of [^{3}H]-thymidine into thecal cells treated with IGF-I, with IGF-I and LH, or IGF-I and FSH (Figure 4.13). Similarly no difference in the incorporation of [^{3}H]-thymidine were seen in granulosa cells treated with IGF-I or IGF-I and FSH (Figure 4.12). However, [^{3}H]-thymidine incorporation into granulosa cells treated with IGF-I and LH combined was significantly greater than the incorporation into granulosa cells treated with IGF-I or LH alone or to the sum of the separate treatments, $P < 0.001$ (Figure 4.12). Analysis of variance, as described in Section 2.5.1., showed that IGF-I and LH acted synergistically to induce DNA synthesis.

4.5.2. A comparison of the effects of IGF-I and oLH or cLH on steroidogenesis and DNA synthesis in granulosa cells.

Granulosa cells obtained from two different birds were prepared and cultured separately, for two parallel experiments. In the first experiment, cells were treated

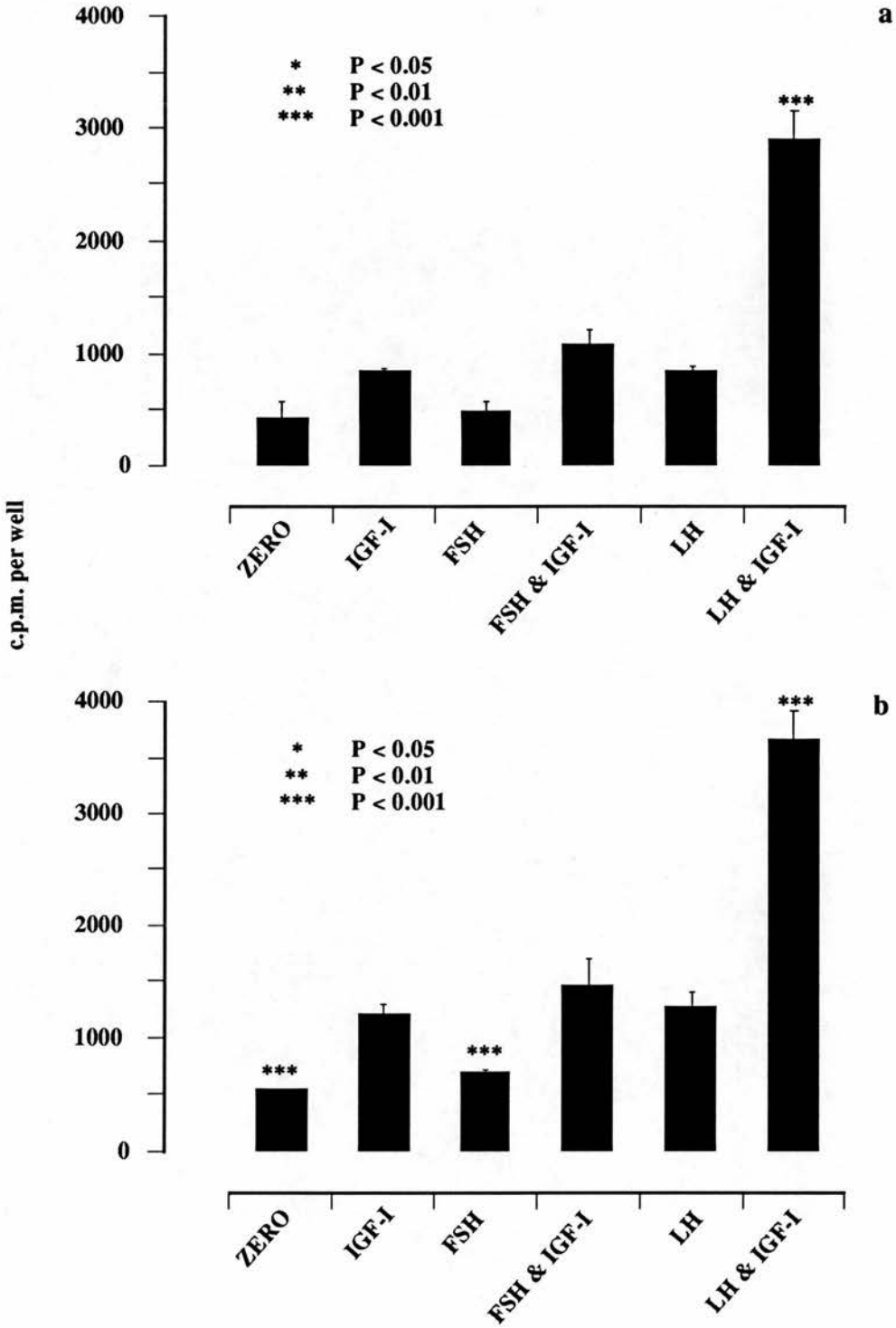

Figure 4.12. Mean uptake of [^{3}H]-thymidine by triplicate granulosa cell cultures in two separate experiments (a and b). Cells were treated as shown (the dose was 25 ng/ml for each treatment). Vertical lines show the standard deviation of the mean. Significance of differences to IGF-I treated cells are indicated.

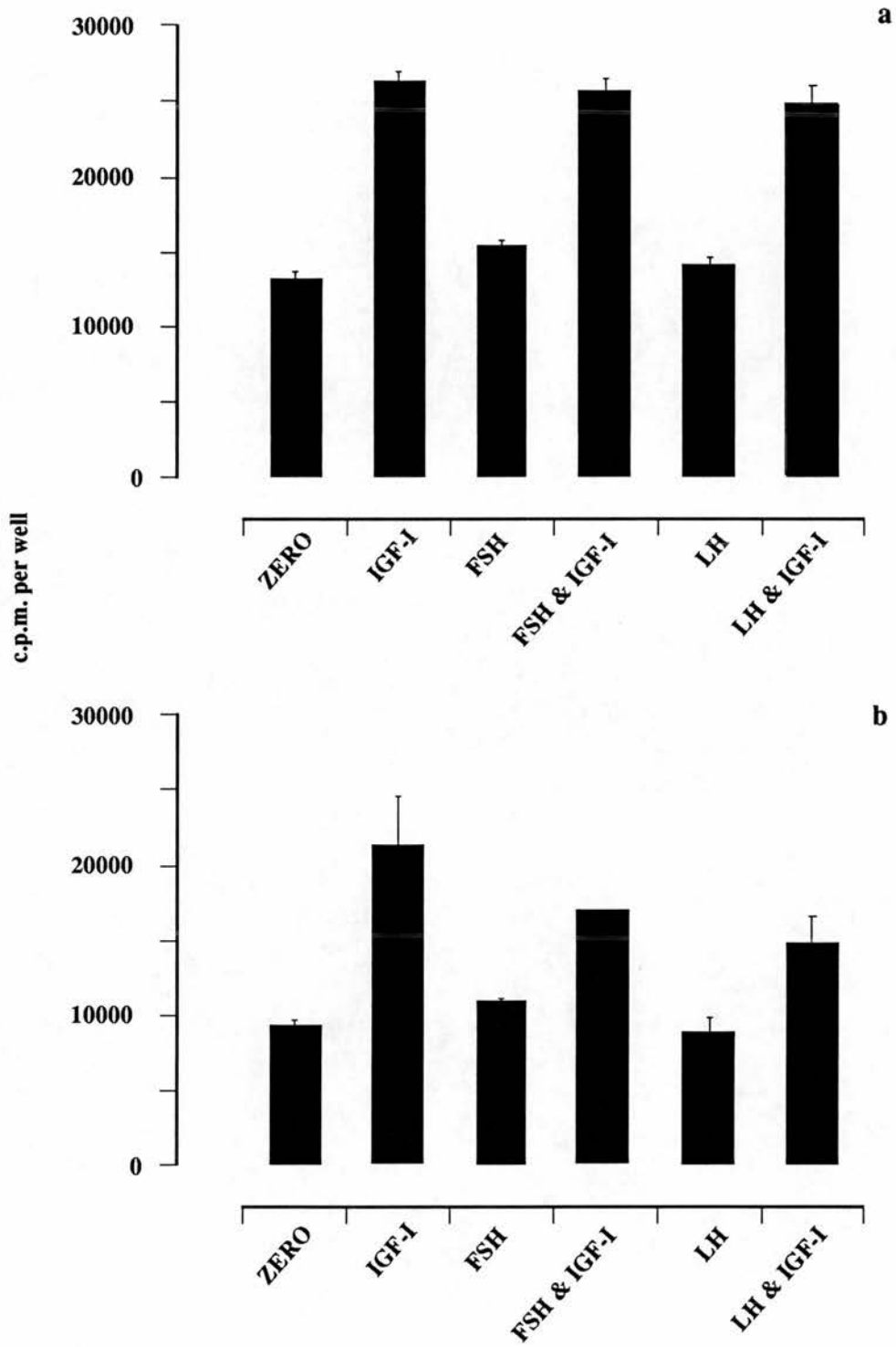

Figure 4.13. Mean uptake of [^{3}H]-thymidine by triplicate thecal cell cultures in two separate experiments (a and b). Cells were treated as shown (the dose was 25 ng/ml for each treatment). Vertical lines show the standard deviation of the mean. Significance of differences to IGF-I treated cells are indicated.

with IGF-I and cLH separately and together at doses of 25 ng/ml and measurement was made of the incorporation of [^{3}H]-thymidine into the DNA. After the treatment period the cell-conditioned media from the cultures were analysed by r.i.a. to determine the progesterone concentrations. In the second experiment cells were subjected to the same protocol as those in the first with the exception that oLH replaced cLH in the treatments.

In both experiments, IGF-I and LH stimulated the incorporation of [^{3}H]-thymidine into granulosa cells as observed in the previous experiment (Section 4.5.1.). Incorporation of [^{3}H]-thymidine stimulated by IGF-I and cLH together was significantly greater than incorporation stimulated by IGF-I alone ($P < 0.001$) (Figure 4.14 a) as was the incorporation due to IGF-I with oLH ($P < 0.001$) (Figure 4.14 b). Analysis of variance (see Section 2.5.1) showed that both oLH and cLH acted synergistically with IGF-I to stimulate the uptake of [^{3}H]-thymidine.

The medium of cells conditioned by granulosa cells treated with oLH or cLH contained significantly more progesterone than medium from the untreated control cultures ($P < 0.001$ in both cases) (Figure 4.15). Medium from cells treated with IGF-I contained no more progesterone than the control medium in both experiments. The concentrations of progesterone in medium conditioned by cells treated with LH was not significantly different from that conditioned by cells treated with LH and IGF-I. There was no evidence for a difference in the response of granulosa cells to oLH or cLH (Figures 4.14, 4.15).

4.6. The Effects Of Anti-LH Antisera On The Uptake Of [^{3}H]-Thymidine By Granulosa Cells

Experiments were conducted to try and confirm the synergistic role of LH with IGF-I in the induction of DNA synthesis. Polyclonal anti-LH antisera treatments were used to immunoneutralize LH. Details of the antisera and the dilutions at which they were used are given in Section 2.4.5.

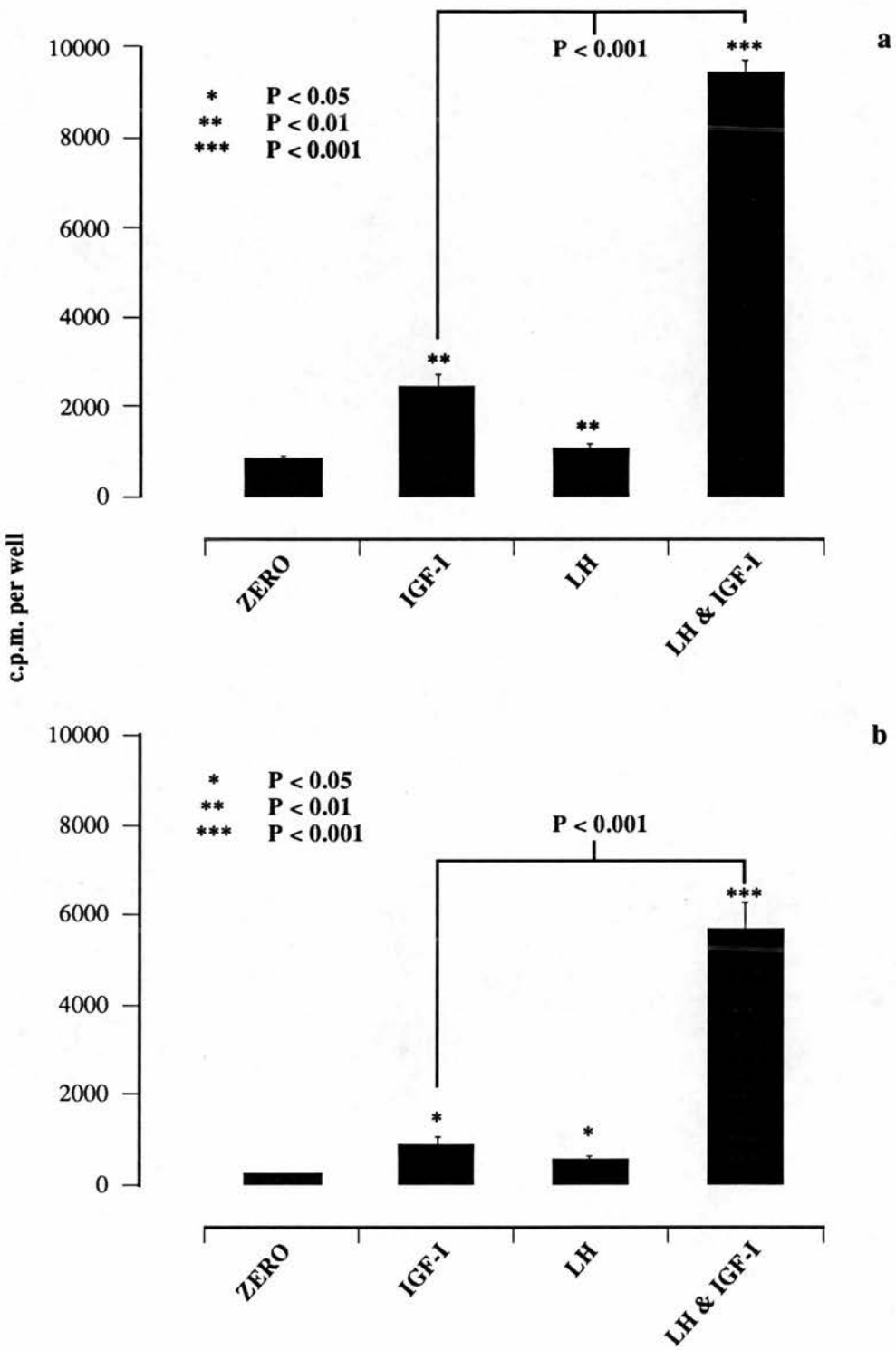

Figure 4.14. Mean uptake of [^{3}H]-thymidine by triplicate granulosa cell cultures in two separate experiments (a and b). Cells were cultured in the presence of cLH and/or IGF-I (a) or with oLH and/or IGF-I (b) (the dose was 25 ng/ml for each treatment). Vertical lines show the standard deviation of the mean. Significant differences between zero and the other treatments are indicated.

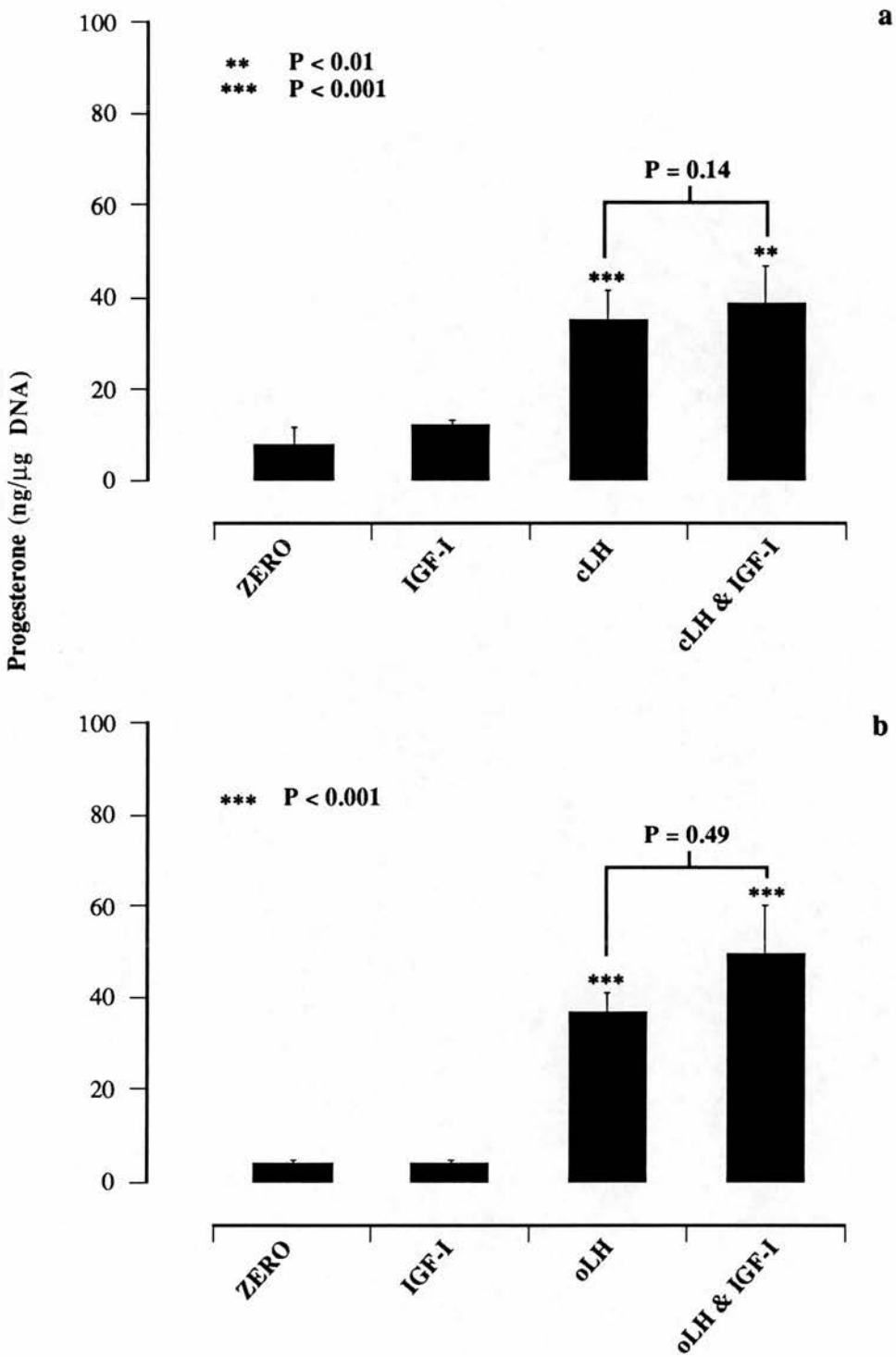

Figure 4.15 Mean progesterone concentrations in culture medium conditioned by granulosa cells cultured in triplicate in two separate experiments (a and b). In **a** Cells were treated with cLH and/or IGF-I (a) or with oLH and/or IGF-I (b) (the dose was 25 ng/ml for each treatment). Concentrations were measured by radioimmunoassay. Vertical lines show the standard deviation of the mean value for each treatment. Significant differences between zero and the others treatments are indicated.

In the first experiment, granulosa cells prepared from the three largest pre-ovulatory follicles were cultured following the protocol described in Section 4.2.3. The first group of cells were untreated or treated with IGF-I alone, LH alone, or with IGF-I and LH together. The dose of IGF-I and LH used was 25 ng/ml for both, as described in Section 4.5.1. In the second group the cells were subjected to the same treatments with the addition of anti LH antiserum. Two experiments were conducted in parallel, the first with oLH and anti-oLH antiserum and the second with cLH and anti-cLH antiserum. The effects of these treatments on DNA synthesis by the cells were measured by incorporation of [^{3}H]-thymidine, the steroidogenesis by measuring the progesterone content of the conditioned medium.

IGF-I and oLH stimulated incorporation of [^{3}H]-thymidine into granulosa cells (Figure 4.16 a) as described previously (Section 4.5.1.). Analysis of variance of the results (see Section 2.5.1.) showed that the combined IGF-I and oLH treatments acted synergistically to stimulate [^{3}H]-thymidine uptake.

Incorporation of [^{3}H]-thymidine by granulosa cells treated with oLH and anti oLH antiserum was not significantly different from that in cells treated with oLH alone (Figure 4.16 a), however the incorporation in cells treated with IGF-I and oLH together was significantly attenuated in comparison with cells to which the antiserum was not added ($P < 0.05$). The most significant effect of antiserum treatment was the greater stimulation of incorporation into cells treated with IGF-I and antiserum, than into cells treated with IGF-I alone ($P < 0.01$).

Treatment of granulosa cells with oLH stimulated the secretion of more progesterone into the culture medium than that secreted by control cultures ($P < 0.0001$) (Figure 4.17 a). Treatment with IGF-I did not stimulate progesterone secretion. This confirms the findings described previously (Section 4.5.2.). It was also confirmed that treatment of granulosa cells with IGF-I and LH together did not stimulate more progesterone release than that induced by treatment with LH alone ($P = 0.7913$). Progesterone production by cells treated with oLH and anti oLH antiserum was significantly less than that by cells treated with oLH alone (P

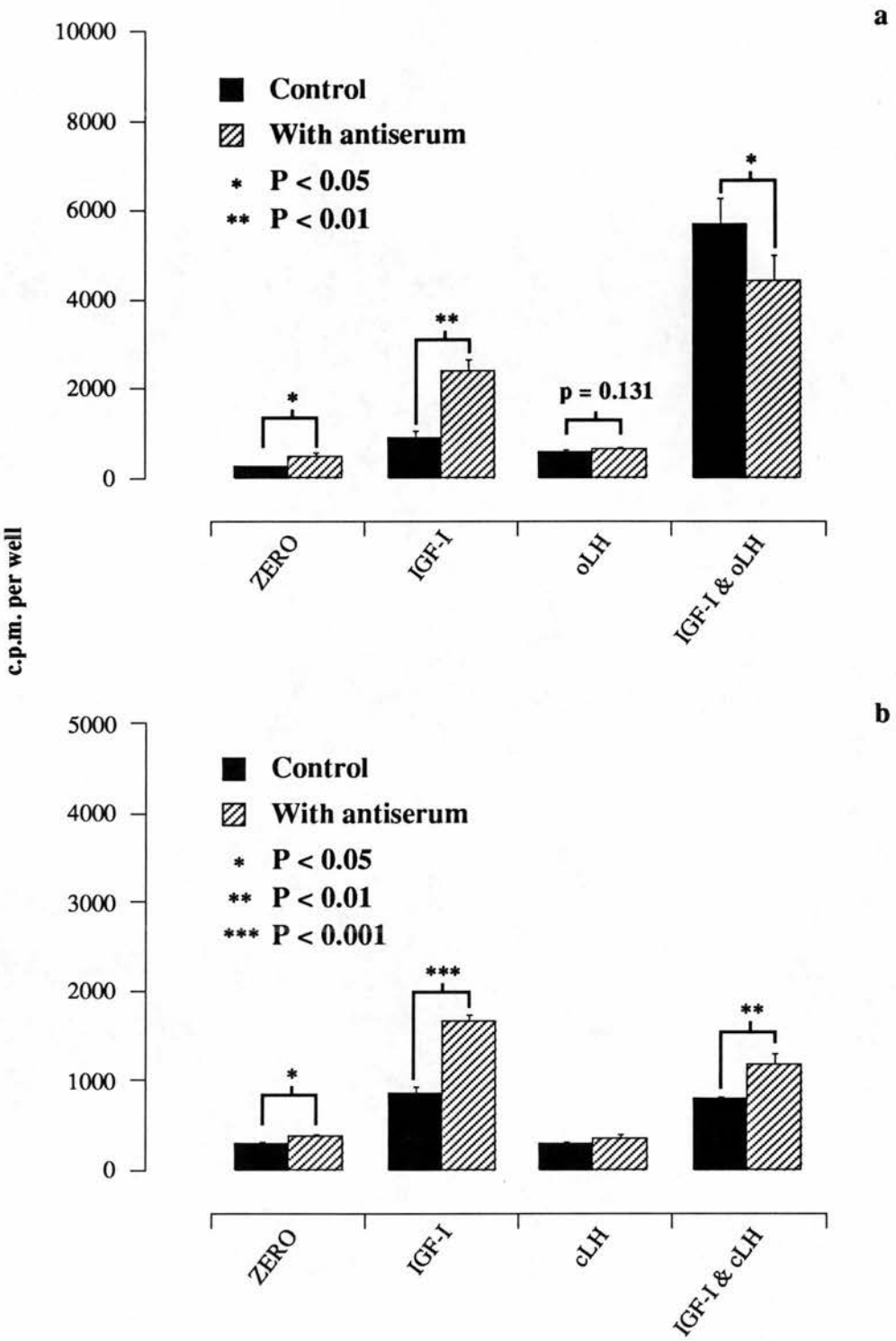

Figure 4.16. Uptake of [^{3}H]-thymidine by granulosa cells treated with LH and/or IGF-I in the presence or absence (control) of anti-LH antiserum as shown (each treatment 25 ng/ml) . Both ovine (o)LH (a) and chicken (c)LH preparations (b) were used. Columns represent the means of triplicate values. Significant differences between the control and antiserum-treated cultures are indicated by asterisks.

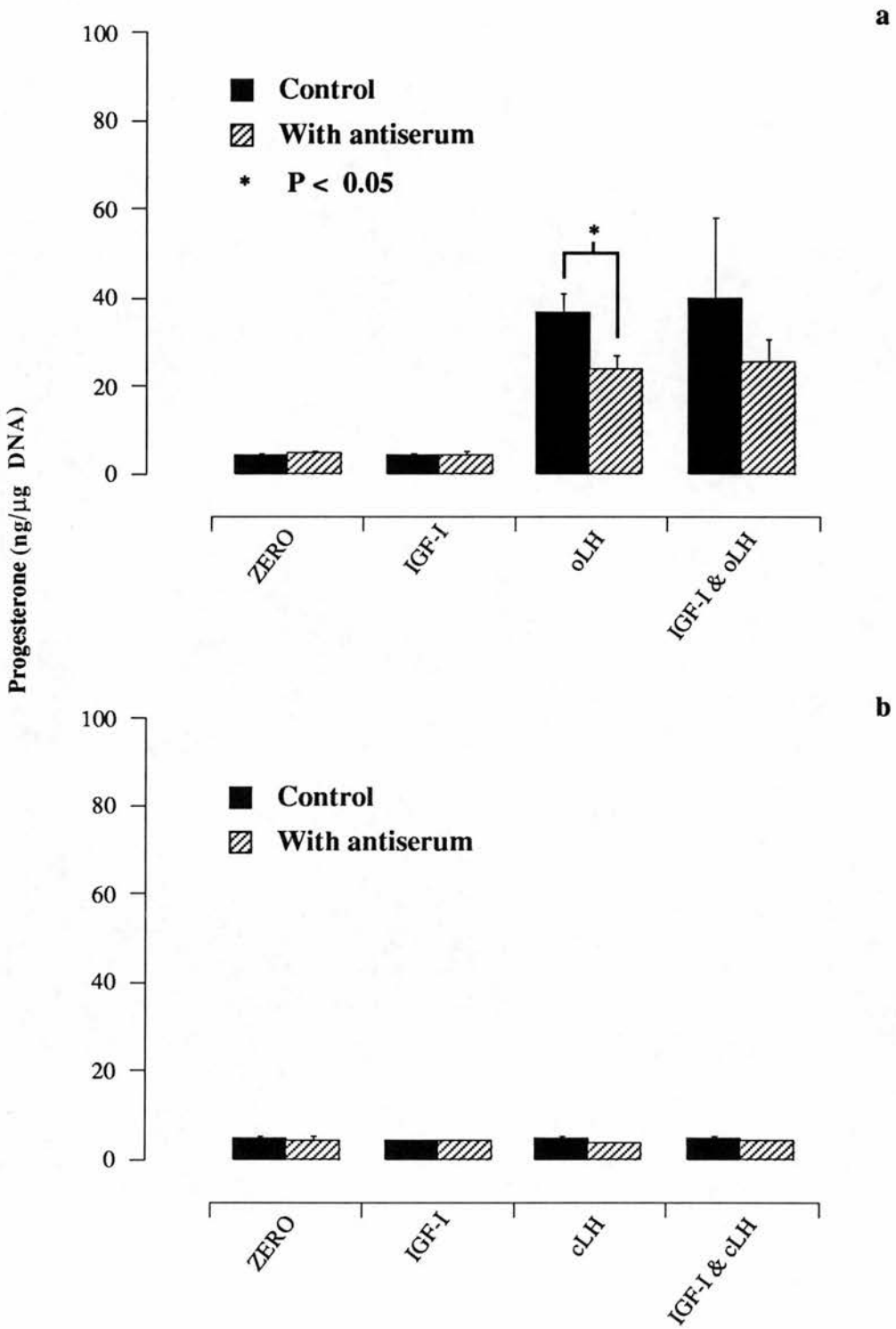

Figure 4.17. Progesterone concentrations in culture medium conditioned by granulosa cells treated with LH and/or IGF-I in the presence or absence (control) of anti-LH antiserum (each treatment 25 ng/ml). Both ovine (o)LH (a) and chicken (c)LH preparations (b) were used. Concentrations were measured by radioimmunoassay. Columns represent the means of triplicate values. Significant differences between the control and antiserum-treated cultures are indicated.

< 0.05). The antiserum did not decrease the production of progesterone by granulosa cells treated with LH and IGF-I together (Figure 4. 17 a).

Experiments with cLH and IGF-I showed that IGF-I, but not cLH significantly stimulated the incorporation of [^{3}H]-thymidine into granulosa cells comparison with the untreated control cells (Figure 4.16b). Analysis of the conditioned medium showed that the cLH preparation did not stimulate progesterone production from these cells either, suggesting that it was biologically inactive. However, the addition of anti-cLH antiserum significantly enhanced the increase in [^{3}H]-thymidine incorporation induced by IGF-I ($P < 0.001$) (Figure 4.16 b). This confirms the observation that treatment with IGF-I and anti-oLH antiserum enhanced [^{3}H]-thymidine uptake (Figure 4.16 a).

The synergistic stimulatory effects of IGF-I and LH antisera on the uptake of [^{3}H]-thymidine by granulosa cells was investigated further, cultured granulosa cells were treated with IGF-I, LH or IGF-I with LH (25 ng/ml of each); anti-cLH, anti-oLH or non-immune serum (NIS), alone and in combination with IGF-I. NIS was included as a control for the antisera; unstimulated cultures were included as negative controls.

All the treatments caused a significantly greater incorporation of [^{3}H]-thymidine into the cells than observed in the untreated control cultures (Figure 4.18). IGF-I, LH and IGF-I with LH caused similar effects to those seen in the previous experiment (see above). Stimulation caused by treatment with the antisera alone was lower than that caused by either IGF-I or LH. Stimulation caused by NIS was lower than that of the antisera. The combinations of IGF-I with anti-oLH, -cLH or NIS all acted synergistically to enhance the incorporation of [^{3}H]-thymidine, but this synergism was not as great as that between IGF-I and LH. The stimulation of [^{3}H]-thymidine uptake by IGF-I with NIS was not significantly different from that by IGF-I with anti-cLH (Figure 4.18).

The possible synergistic effects of low doses of FCS on [^{3}H]-thymidine incorporation caused by IGF-I in granulosa cells were investigated. Cultured

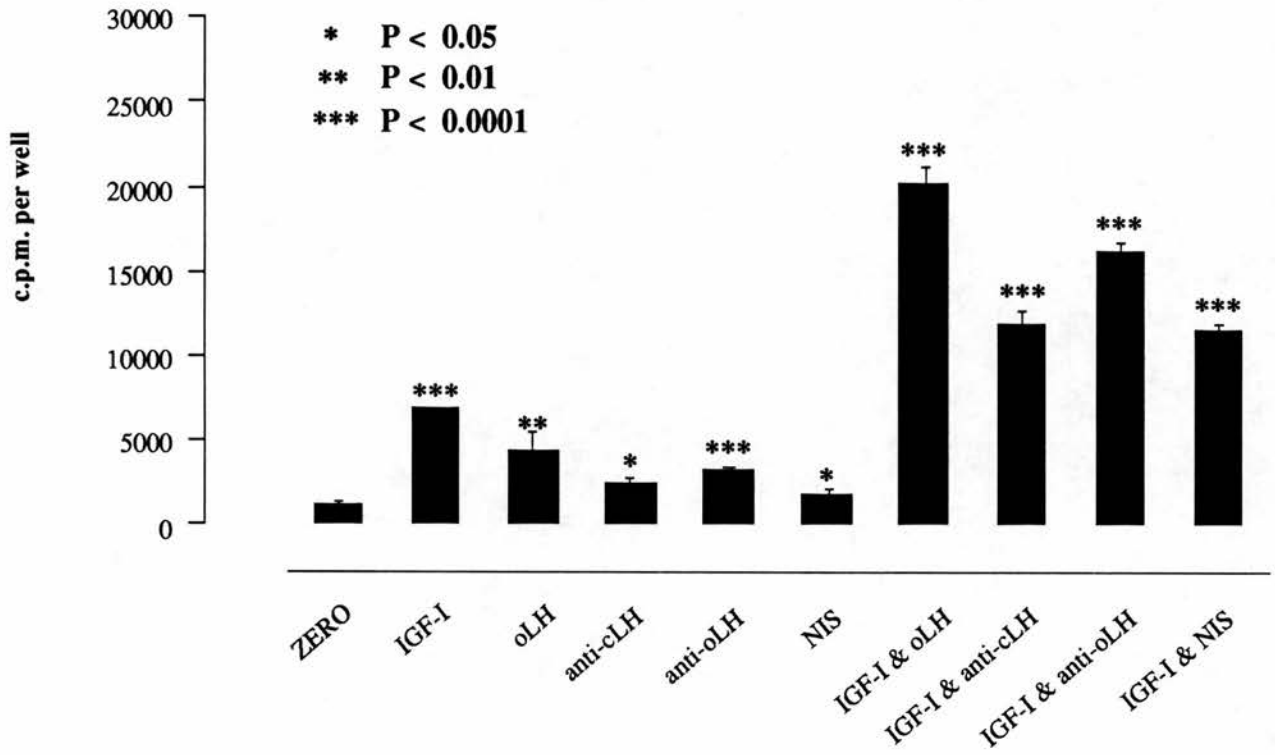

Figure 4.18 Mean uptake of [^{3}H]-thymidine by granulosa cells cultured in triplicate, treated as indicated.Vertical lines show the standard deviation of the mean value for each treatment. Significant differences between zero and the other treatments are indicated.

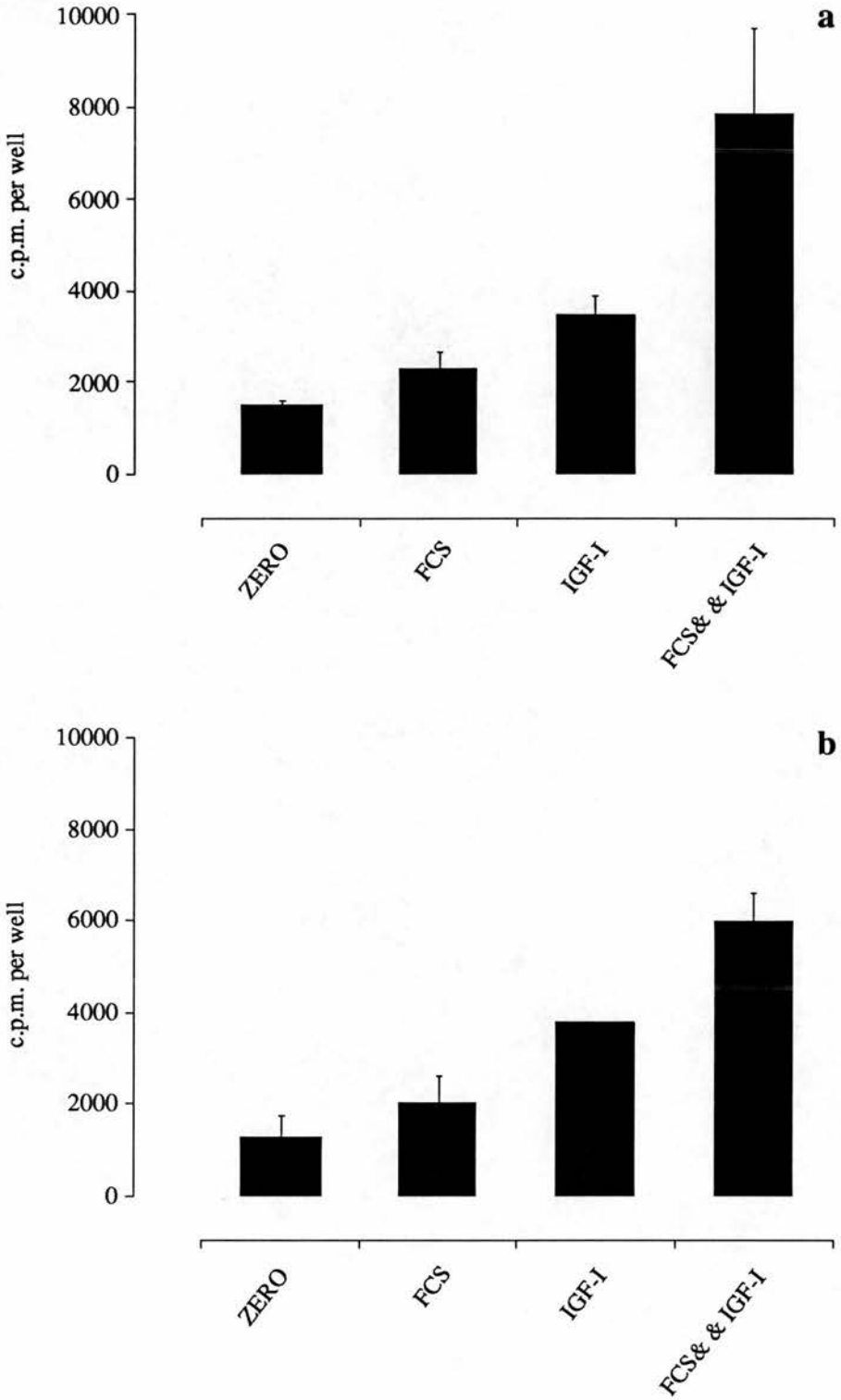

Figure 4.19. The mean incorporation of [^{3}H]-thymidine into triplicate granulosa cell cultures, untreated (zero) or treated with IGF-I (25 ng/ml) and FCS (0.1%, v/v). Standard deviations are shown as vertical bars. These are the results of two independent experiments (a and b).

granulosa cells were treated with IGF-I (25 ng/ml), FCS (0.1% v/v) or a combination of both; untreated cultures were used as controls; the experiment was replicated. FCS (0.1%) acted synergistically with IGF-I to enhance the incorporation of [^{3}H]-thymidine into granulosa cells in both experiments (Figure 4.19).

In summary, the results of the three experiments described in this section show that the method used to negate the effects of LH on the uptake of [^{3}H]-thymidine with antisera was not effective, but that the treatment enhanced the stimulatory effects of IGF-I on granulosa cells. This result was confirmed in the second experiment, which also showed (as did the third experiment) that treatment of cells with non-immune serum or fetal calf serum resulted in stimulation of [^{3}H]-thymidine incorporation in a similar manner to anti-LH antisera.

4.7. Developmentally Related Differences In The Response Of Granulosa And Thecal Cells To IGF-I And Gonadotrophins

Experiments were conducted with cells from the largest ovarian follicles in the hierarchy. Granulosa and thecal cells from the largest (F1), third largest (F3) and fifth largest (F5) follicles were used. Material from follicles at the same stages of the ovulatory cycle was pooled and prepared for culture. The response of the cultures to the treatments was measured by the incorporation of [^{3}H]-thymidine into the DNA of the cells.

4.7.1. IGF-I treatment of cells at different stages of development

Granulosa and thecal cells from the ovaries of two laying hens were set up in culture. The cultures were treated in triplicate with IGF-I for 24 hours, with doses ranging from 1.5 to 50 ng/ml, untreated cultures were included as controls. The uptake of [^{3}H]-thymidine by the cells during treatment was measured and the results expressed as c.p.m. per well. These experiments were repeated twice

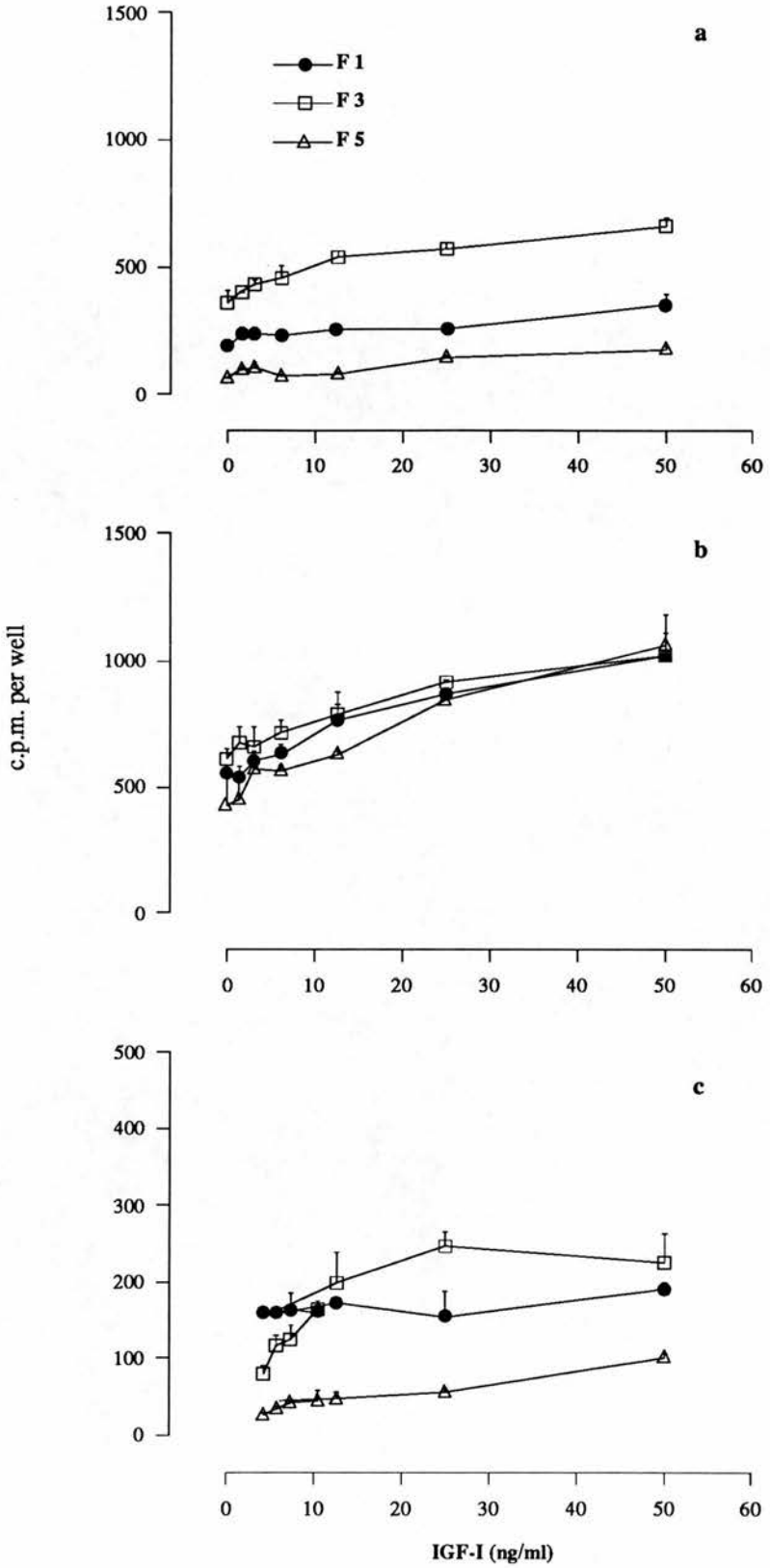

Figure 4.20 Mean uptake of [^{3}H]-thymidine by triplicate granulosa cell cultures from F1, F3 and F5 follicles in three independent experiments (a,b and c). Cells were treated with IGF-I at the doses indicated for 24 hours. Standard deviations of the mean are shown as vertical bars.

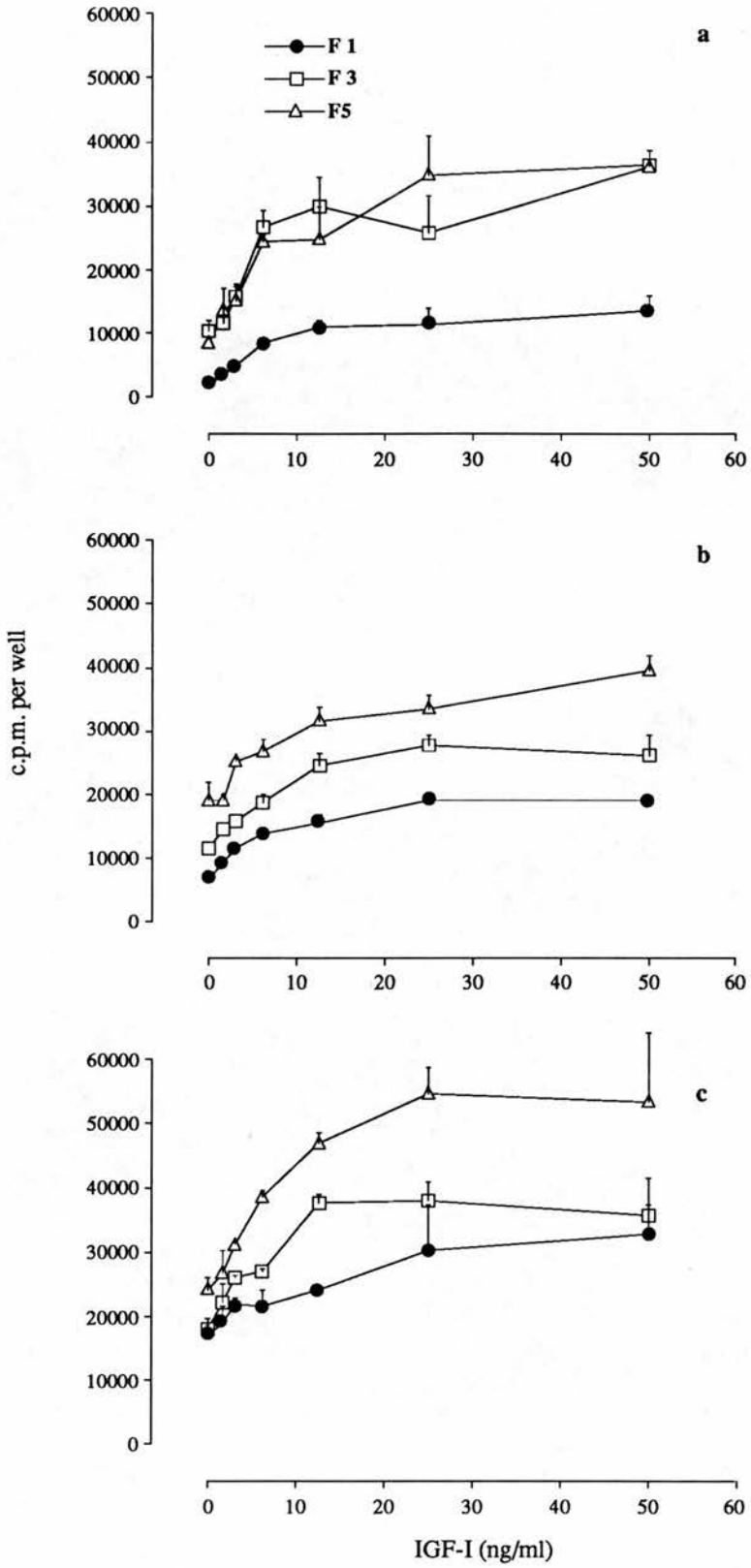

Figure 4.21 Mean uptake of [^{3}H]-thymidine by triplicate thecal cell cultures from F1, F3 and F5 follicles in three independent experiments (a,b and c). Cells were treated with IGF-I at the doses indicated for 24 hours. Standard deviations of the mean are expressed as vertical bars.

each, the experiments for each cell type are labelled a, b and c (Figures 4.20. and 4.21.). Additionally, the mean values for each follicle size were themselves meaned for the three thecal and the three granulosa cell experiments (Figure 4.23). The granulosa data was first normalised with respect to the uptake of [^{3}H]-thymidine by untreated cells from the F1 follicle, since the range of values in the three experiments did not overlap sufficiently to allow a direct comparison. This was done by dividing the value of each data point by the value of the unstimulated F1 cultures in each experiment.

Granulosa cells from the smallest follicles (F5) incorporated less [^{3}H]-thymidine than those from F3 or F1 follicles; however this difference was not significant over the whole IGF-I dose range in the third experiment (c). The results also indicated that [^{3}H]-thymidine uptake was greatest by cells from F3 follicles; however, this was only significant in the first experiment (a) (Figure 4.20).

In two of the three experiments (b and c) thecal cells incorporated [^{3}H]-thymidine in the order F5>F3>F1, in the first experiment (a) the uptake by F3 and F5 cells were not significantly different from each other, but both were significantly greater than that by the F1 cells.

The dose-response curves were compared in each experiment by the ALLFIT statistical package (Table 4.2). There were no significant differences in the shapes of the curves from F1, F3 and F5 cultures of either cell type. The ED_{50} values for IGF-I were in the range of 4.07 to 8.68 for thecal cultures and 4.75 to 12.05 for granulosa cultures.

Overall, the results of these experiments indicate that granulosa and thecal cells respectively incorporate [^{3}H]-thymidine in the orders F3 > F1 > F5 and F5 > F3 > F1 in response to stimulation by IGF-I. However, no significant differences in the dose-response curves due to follicle size were detected.

Table 4.2 The ED_{50} values of IGF-I with respect to the stimulation of [^{3}H]-thymidine incorporation into granulosa and thecal cells in three independent experiments. In each experiment the effect on cells from F1, F3 and F5 follicles was examined..

Experiment No.	**Follicle Type**	**ED_{50} (± SEM)**	
		Thecal cells	Granulosa cells
1	F 1	6.14 ± 4.83	4.75 ± 1.14
	F 3	4.07 ± 0.99	6.58 ± 0.84
	F 5	4.20 ± 1.45	6.25 ± 4.83
2	F 1	4.54 ± 1.56	4.23 ± 2.11
	F 3	5.91 ± 1.32	5.63 ± 1.50
	F 5	3.67 ± 0.67	5.35 ± 7.69
3	F 1	8.68 ± 7.61	11.21 ± 4.66
	F 3	5.18 ± 1.75	12.05 ± 6.13
	F 5	5.45 ± 1.08	6.67 ± 1.46

Table 4.3 The ED_{50} values of LH with respect to the stimulation of [^{3}H]-thymidine incorporation into granulosa cells in three independent experiments. In each experiment the effect on cells from F1, F3 and F5 follicles was examined..

Experiment No.	**Follicle Type**	**ED_{50} (± SEM)**
1	F 1	9.54 ± 0.656
	F 3	5.6 ± 0.173
	F 5	16.35 ± 1.85
2	F 1	12.69 ± 0.57
	F 3	11.64 ± 1.08
	F 5	13.22 ± 7.22
3	F 1	8.22 ± 1.3
	F 3	7.37 ± 0.24
	F 5	8.3 ± 0.12

4.7.2. Gonadotrophin treatment of cells at different stages of development

Granulosa and thecal cell cultures were set up from follicles at different developmental stages as described in the preceding section. The cultures were untreated, or treated with LH and FSH separately at doses of 5, 25 or 50 ng/ml; cultures were also treated with IGF-I with and without LH or FSH (all treatments added at 25 ng/ml). The effect of treatments on the [^{3}H]-thymidine incorporation by the cells was measured; this experiment was repeated twice.

FSH did not stimulate [^{3}H]-thymidine uptake in either cell type; LH stimulated uptake by granulosa cells (at all three stages of development), but not by thecal cells. These results are similar to those described previously (Section 4.5.), in which experiments, cells were pooled from follicles of different developmental stages. LH stimulated [^{3}H]-thymidine incorporation in a dose-dependent manner for granulosa cells from all follicle types in all three experiments (Figure 4.22). The ED_{50} values were calculated for each dose response curve shown in Figure 4.23 using the ALLFIT statistical program, these values are shown in Table 4.3. The results of this analysis showed that there was no significant differences in the ED_{50} values of these curves. The maximum incorporation per F3 culture was significantly greater than that in F1 cultures, which in turn was significantly greater than that in F5 cultures. The mean of the triplicate values for each LH dose in the three experiments were normalised with respect to the untreated F1 value as described in Section 4.7.1. (Figure 4.23c); this provided a comparison of results between experiments. There was no significant difference in the maximum incorporation into F1 and F3 granulosa cells when stimulated with LH; however, the incorporation into F5 cells was significantly less when compared at each dose than that of the other two ($P < 0.05$).

In summary, the results of this experiment show that granulosa cells from follicles at different stages of development respond differently to LH stimulation, and that the order of response is F3 > F1 > F5.

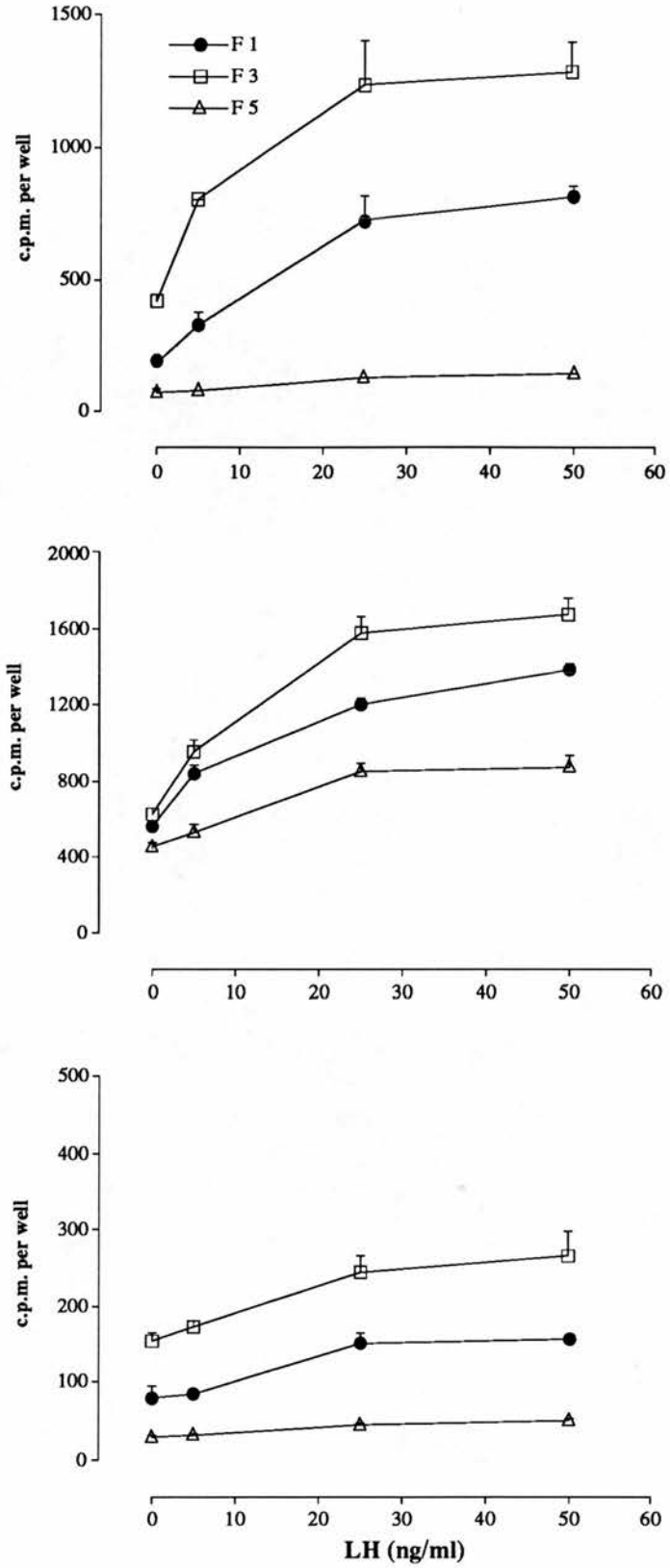

Figure 4.22 Three independant experiments (a,b and c) in which cultured granulosa cells from F1, F3 and F5 follicles were stimulated with LH at the doses indicated for 24 hours. The resulting incorporation of [^{3}H]-thymidine into the cells was measured (c.p.m. per well) and is expressed on the y axis. Each point is the mean of triplicate cultures, standard deviations are expressed as vertical bars.

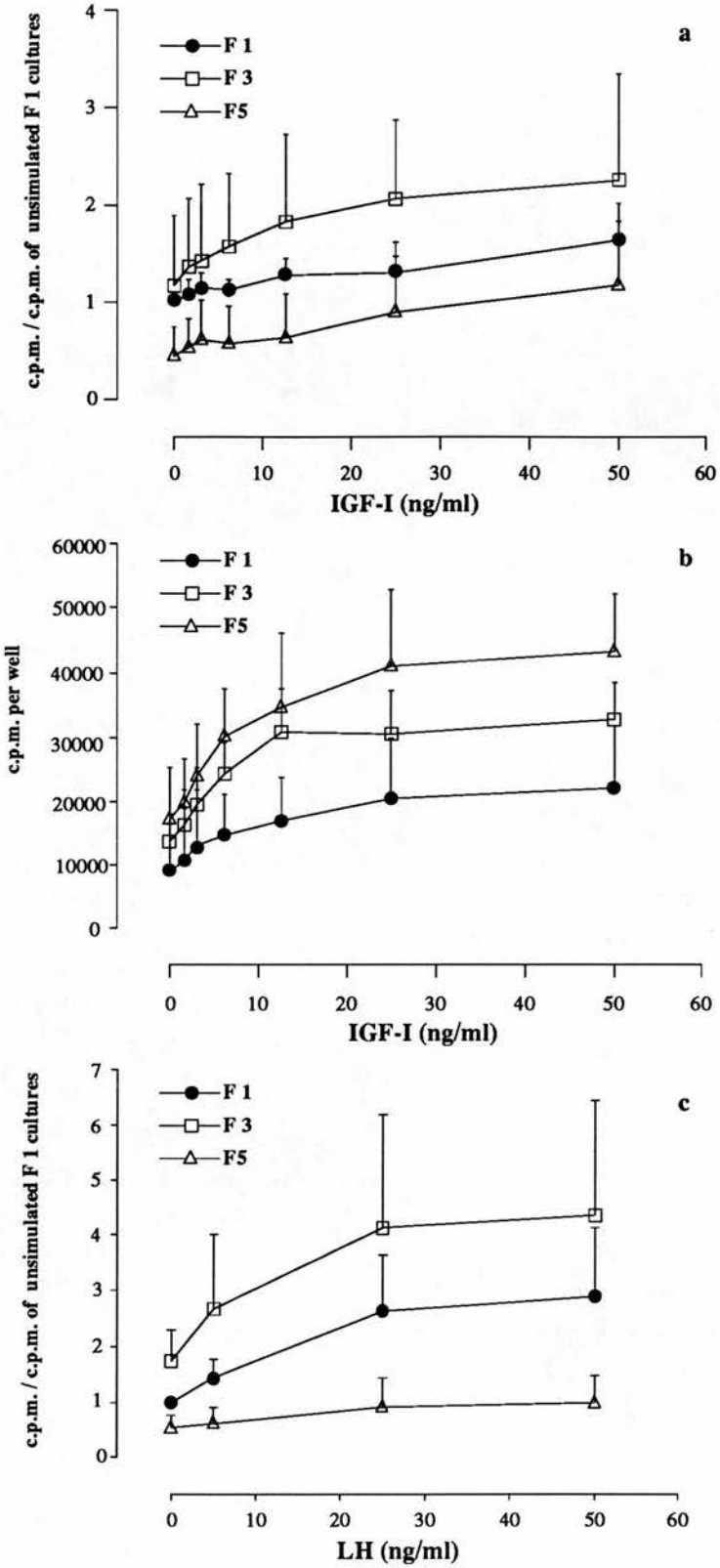

Figure 4.23 Meaned values from three independent experiments measuring the response of cultured granulosa (a) and thecal (b) cells from F1, F3 and F5 follicles to IGF-I treatment, and the response of cultured granulosa cells from F1, F3 and F5 follicles to LH treatment (c). In (a) and (c), the data was normalised to the mean incorporation by the untreated F1 cells in each experiment. The vertical bars represent the standard deviation of the means in each set of three experiments.

In the experiment described above, granulosa cells were treated with IGF-I, LH, LH with IGF-I, FSH or FSH with IGF-I, in each case the treatments were 25 ng/ml. The combined effects of IGF-I and FSH on [^{3}H]-thymidine incorporation in these cells was not significantly different from that of IGF-I treatment alone (results not shown). However, IGF-I and LH treatments were synergistic for cells derived from each follicle type in each of the three experiments.

4.8. Summary And Discussion

Chicken granulosa and thecal cell culture systems were developed to determine the role of IGF-I in the growth of chicken ovarian cells *in vitro*. Protocols for short term cell culture for the measurement of steroid output were available; however, there was a requirement to develop new protocols since the exisiting ones were not suitable for the experiments required. The systems which were developed allowed the culture of cells for at least 4 days with the opportunity to measure DNA synthesis and the steroid output of the cells over the last 24 hours of this period.

Serum contains growth factors (including EGF, NGF, the IGFs and PDGF), nutrients, attachment proteins and transport proteins which are required by cells for growth *in vitro* (Temin, 1971; Gospodarowicz and Moran, 1976). Serum was added to cultures of granulosa and thecal cells and its effect on DNA synthesis was measured (Section 4.3), demonstrating the ability of cells in the culture system to respond to growth stimuli. The results of these experiments showed that cells of both types responded positively to serum stimulation and in a dose-dependent manner.

The steroidogenic responses of chicken granulosa and thecal cells *in vitro* are well established (see Chapter 1). For example, Robinson *et al.* (1988) showed that LH and FSH stimulate progesterone and androstenedione production by cultured granulosa and thecal cells, respectively. The results described in Section 4.4.2 confirmed these observations.

The cell culture systems were thus shown to be responsive to stimuli intended to induce cell growth and steroidogenesis. Hence, the systems were validated for measuring these parameters in granulosa and thecal cells *in vitro*.

The remaining experiments in this chapter used the cell culture systems to study the effects of IGF-I and the gonadotrophins (LH and FSH) on both cell types. The primary aim of the experiments was to examine the effect of treatments on cell growth, steroidogenesis was measured to confirm the biological activity of the gonadotrophins.

IGF-I has well-documented mitogenic effects on virtually all cell types, evidence for this activity was reviewed in Chapter 1. The experiments in Section 4.4.1 showed that IGF-I stimulates DNA synthesis in avian granulosa and thecal cells *in vitro* and that this occurs in a dose-dependent manner, the range of doses used being physiologically significant (Hammond *et al.*, 1985). The maximum synthesis resulting from stimulation was greater in thecal cell cutures than granulosa cultures, but more cells were plated in the thecal cultures. When the stimulation per plated cell was calculated, it remained greater for thecal cells. This shows that differences exist in the response of granulosa and thecal cells to IGF-I, the thecal cells demonstrating a greater response. A possible explanation for the differences in DNA synthesis between cell types is that the theca is a faster growing tissue than the granulosa *in vitro*.

The effect of IGF-I in these experiments indicates that it is involved in the process of growth in both granulosa and thecal cells. Work by Leof *et al.* (1982) and Campisi and Pardee (1984) has shown that IGF-I is one of many factors that have been identified as a requirement for the functions of the cell cycle - the sequential processes of DNA replication, mitosis and cell division. Therefore, the demonstration of IGF-I's ability to stimulate DNA synthesis is consistent with a role in the mitosis of granulosa and thecal cells. However, no study (including this one) has investigated the complex area of cell cycle control in chicken ovarian cells, therefore the specific role of IGF-I with respect to the growth of these cells is not clear. A study of the effects of IGF-I on cell cycle events in these cells may clarify the role that it has on growth regulation of chicken ovarian cells.

The results of experiments described in Section 4.4.3 show that LH has an effect on DNA synthesis in granulosa cells which is similar to that observed for IGF-I in the experiments described above. However, this effect was not seen with thecal cells. These cell-specific effects show that clear differences exist in the growth stimulation pathways of thecal and granulosa cells with respect to LH action and further suggest that its effects on growth are not mediated by the same pathway as that used by IGF-I. In contrast to LH, FSH had no effect on DNA synthesis in either granulosa or thecal cells *in vitro*.

Gonadotrophins have previously been shown to stimulate the growth of chicken granulosa cells *in vitro* (Yoshimura and Tamura, 1988), corroborating the results in Section 4.4.3. Further work has shown that when dibutyryl(db) cAMP is added to chicken granulosa cell cultures, it causes cell proliferation (Yoshimura and Tamura, 1991). The implication of that work is that the mitogenic effects of LH observed in this study and by the studies described above, are mediated by cAMP since it is well established that this molecule mediates other gonadotrophin-induced effects (steroidogenesis) in mammalian ovarian cells (Richards *et al.*, 1979; Richards, 1980). The lack of similar effects with FSH contrast with the work of Yoshimura and Tamura (1988) which showed that FSH had the same effects as LH with respect to granulosa cell proliferation; In the latter case the cells were cultured for 7 days, whereas in those decribed in 4.4.3 the changes over 24 hours of culture were measured. Thus it is possible that FSH might not be as effective as LH in the shorter term cultures.

The biological activity of the FSH preparation used in these experiments was demonstrated by its ability to elicit an androstenedione production response from chicken thecal cells (Section 4.4.2); however, this is an ovine preparation and no comparison has been made with effects on DNA synthesis of a chicken FSH (pure cFSH was not available). Therefore it is possible that while ovine FSH is ineffective, the chicken species may be potent in this respect. However, both cLH and oLH were shown to elicit very similar responses from granulosa cells in these experiments with repect to both DNA synthesis and progesterone production (Section 4.5.2).

Following the discovery that both IGF-I and LH stimulated granulosa cell DNA synthesis in a similar manner, the possibility of additive or synergistic interactions between IGF-I and gonadotrophins was investigated. The results were described in Section 4.5. FSH was found to have no effect on IGF-I-mediated DNA synthesis in either granulosa or thecal cell cultures, and LH had no effect in this respect on thecal cell cultures, confirming the results in Section 4.4.3. However, LH and IGF-I were synergistic with respect to DNA synthesis in granulosa cell cultures, but not with respect to progesterone output. The results indicate that the mitogenic and steroidogenic activities of LH are mutually exclusive and that IGF-I does not affect the latter. Mammalian granulosa cells have been shown to respond to IGF-I treatment by producing pregnenolone in a time-dependent manner (Veldhuis *et al.*, 1986), with pronounced effects not apparent until 62-84 hours of treatment. These experiments were conducted with a treatment period of 24 hours; therefore, it is possible that extending the period for which cells are treated with IGF-I and LH could elicit a synergistic effect with respect to progesterone synthesis, similar to that reported for the effects IGF-I and FSH on several steroidogenic functions of mammalian granulosa cells (Adashi *et al.*, 1985b; 1988a).

Experiments were conducted to investigate the mechanisms of the synergy observed between IGF-I and LH. Initially, an attempt was made to eliminate the synergy by using anti-LH antiserum; this did not affect the DNA synthesis of cells in any of the LH-treatment groups but had a synergistic action with IGF-I similar to that observed with LH. This experiment led to speculation that the antiserum effects were serum-mediated, since serum contains many mitogens which could potentially act in a synergistic manner with IGF-I. To investigate this, the second experiment compared the effects of two separate LH-antisera and non-immune serum (NIS) on the mitogenic effects of IGF-I. This experiment showed that there were no significant differences in the three treatments. A third experiment showed that 0.1% FCS was similarly synergistic with IGF-I.

The conclusions drawn from the three experiments described above, were that serum has a synergistic effect with IGF-I on DNA synthesis in granulosa cells, and that the effects of antisera were due to the presence of serum rather than antibodies. The results indicate that serum and LH act via a common pathway with respect to their synergism with IGF-I since serum significantly stimulated [^{3}H]-thymidine uptake by granulosa cells above the level observed in unstimulated cultures, but did not have a significant effect on the uptake of [^{3}H]-thymidine due to LH. One possible mechanism suggested by these results is that LH synergy could be mediated by secondary factors produced by granulosa cells into the culture medium, and that these factors are also present in serum. One candidate for such a factor is progesterone, which was shown to be produced by granulosa cells in response to LH; it is also present in low concentrations in the serum used (100 pg/ml) according to the suppliers (Sigma Chemical Co.). However, this could not be confirmed in this study since the progesterone r.i.a. available has a sensitivity limit of 100pg/ml. A direct role of LH on the cells could not be eliminated since the serum used contained LH (900 pg/ml) according to the suppliers (Sigma Chemical Co.). Another candidate for an intermediate role is EGF which is present in serum and has been shown to promote chicken granulosa cell proliferation *in vitro* (Yoshimura and Tamura, 1988; Peddie, 1992).

The existence of a developmental hierarchy in the domestic hen in which follicles exist in well-defined developmental states has prompted much speculation into how it is regulated. One possible explanation is that most follicles are susceptible to atresia, but follicles destined to ovulate may be sensitised to the effects of growth factors and thus able to develop. The involvement of gonadotrophins in such a mechanism has been suggested by work in human ovaries, showing that FSH concentrations in follicular fluid are different in follicles destined to ovulate compared with those in smaller follicles (McNatty *et al.*, 1975). It has has also been shown that IGF-I and gonadotrophins have synergistic effects in avian (this study) and mammalian (Adashi *et al.*, 1985b) follicles. Therefore, there is a possibility that LH may sensitise the cells of ovarian follicles in the domestic hen to the effects of IGF-I and possibly other growth

factors. Recent work shows that endonuclease enzyme activity mediates follicular atresia in the avian ovary (Tilly *et al.*, 1991) and suggests that factors, possibly gonadotrophins or growth factors, mediate this enzyme activity and thus control atresia. Further study of the response of cells to IGF-I / gonadotrophins from follicles over a much greater developmental range may indicate the role(s) of these factors in atretic processes.

Another important conclusion of these experiments is that when designing experiments to investigate IGF-I effects, precautions should be taken to ensure that serum is not used in any significant amounts since it clearly has an effect on the actions of IGF-I.

Further experiments were carried out to investigate the mechanisms of LH and IGF-I synergy with respect to granulosa cells. These involved a study on the effects of BP production by the cells and the effects on receptor binding. These experiments are described in detail in Chapters 5 and 6 respectively.

The final set of experiments described in this Chapter were conducted in order to elucidate the effect of developmental stage of cells on the responses described above. There were no significant differences in the shape of the dose-response curves of the cells from different aged follicles to any of the stimuli and IGF-I and LH were synergistic with respect to the stimulation of DNA synthesis in granulosa cells from all three follicle types. However, the maximum response of cells to IGF-I stimulation was dependent on the developmental stage of the follicle from which they were removed. Thecal cells had an inverse relationship with follicular age in this respect; the maximum response of granulosa cells to either IGF-I or LH was seen in the F3 follicles in both cases and the smallest in the F5 follicles with the response of F1 granulosa cells intermediate between the two.

Thus, developmentally-related changes in the mitogenic response of both granulosa and thecal cells exist, and in thecal cells the response declines with follicle size. The discovery that the order of response of granulosa cells from different follicles is the same for both IGF-I and LH treatments indicates that the mitogenic response is by a

common pathway. The order of response in granulosa cells also indicates that granulosa cells are capable of their maximum response to mitogenic agents at the F3 stage. Developmentally-related changes also occur in the steroidogenic responses of granulosa cells (Bahr *et al.*, 1983); however, these do not appear to correspond with the pattern of growth response discovered here. Any link of the cells' growth and steroidogenic responses would predict a greater growth response of F1 granulosa cells to LH than F3 cells since follicles progress from an FSH to an LH-controlled phase as they approach ovulation (Calvo *et al.*, 1981), the converse was observed here.

To summarise, a tissue culture system was developed which was shown to support mitosis and steroidogenic activity of both cell types. Through this system, IGF-I was shown to stimulate DNA synthesis in both thecal and granulosa cells and LH was shown to stimulate this activity in granulosa cells in a manner similar to IGF-I. When IGF-I and LH treatments were combined they were synergistic with respect to DNA synthesis in granulosa cells, but not with respect to progesterone production. Examination of developmental effects revealed that thecal cells from larger follicles were less responsive to IGF-I, but that granulosa cells were most responsive to IGF-I at the F3 stage of development.

Amongst the conclusions drawn from the work described in this chapter are those that IGF-I has a role in the growth of both granulosa and thecal cells in the domestic hen, and that there are differences in the effects of this peptide *in vitro* which are dependent on the cell type. LH is involved in granulosa but not thecal cell growth, and it has a synergistic action with IGF-I in this respect. Experiments to elucidate the mechanism of this synergy revealed that serum is similar to LH in this respect. However, no conclusions could be drawn from this discovery, but it was speculated that LH may stimulate the production of a factor (or factors) from granulosa cells which act with IGF-I, and that such factors are present in serum. It was also concluded that the growth responses of both granulosa and thecal cells to IGF-I and of granulosa cells to LH are developmentally regulated.

CHAPTER 5: IGF-I BINDING PROTEINS IN THE AVIAN OVARY

5.1. INTRODUCTION

The production of IGF-I binding proteins by chicken granulosa and thecal cells *in vitro* was investigated using the culture systems described in Section 4.2. A series of experiments was conducted which compared endogenous production of IGFBPs with production from cells treated with IGF-I. The influence of IGF-I and LH on IGFBP production by granulosa cells was investigated to determine whether binding proteins are involved in the synergistic effects of IGF-I and LH on the uptake of [^{3}H]-thymidine by chicken granulosa cells (Chapter 4).

The size of the cultures was greater than in other experiments described in order to procure binding protein production by the cells at detectable levels. Therefore, cells were plated on 25 cm^2 area flasks at cell densities of 50,000 and 250,000 cells/cm^2 for granulosa and thecal cells, respectively. No serum was included in the culture medium for the treatment period in order to eliminate the possibility of adding exogenous IGF binding proteins which are present in serum. Cell-conditioned medium samples were concentrated and then run on PAGE gels which were blotted to nitrocellulose membranes and incubated with [^{125}I]-IGF-I.

5.2. PRODUCTION OF IGFBPS BY GRANULOSA CELLS *IN VITRO*

Granulosa cells were treated with IGF-I (25ng/ml), LH (25ng/ml) or IGF-I and LH (25/ng/ml of both). Analysis of the resulting cell-conditioned medium (GCM) showed that the radioactive ligand had bound to five regions in each sample track (Figure 5.1 a). When compared with the marker proteins, these bands were found to correspond to proteins with approximate molecular weights of 32.5, 31.5, 30.5, 29.5 and 24.5 kDa. Measurement of the intensity of these bands (Shimadzu densitometer, not shown) revealed differences in the band intensities between samples. The treatment of cells with IGF-I increased the amounts of all

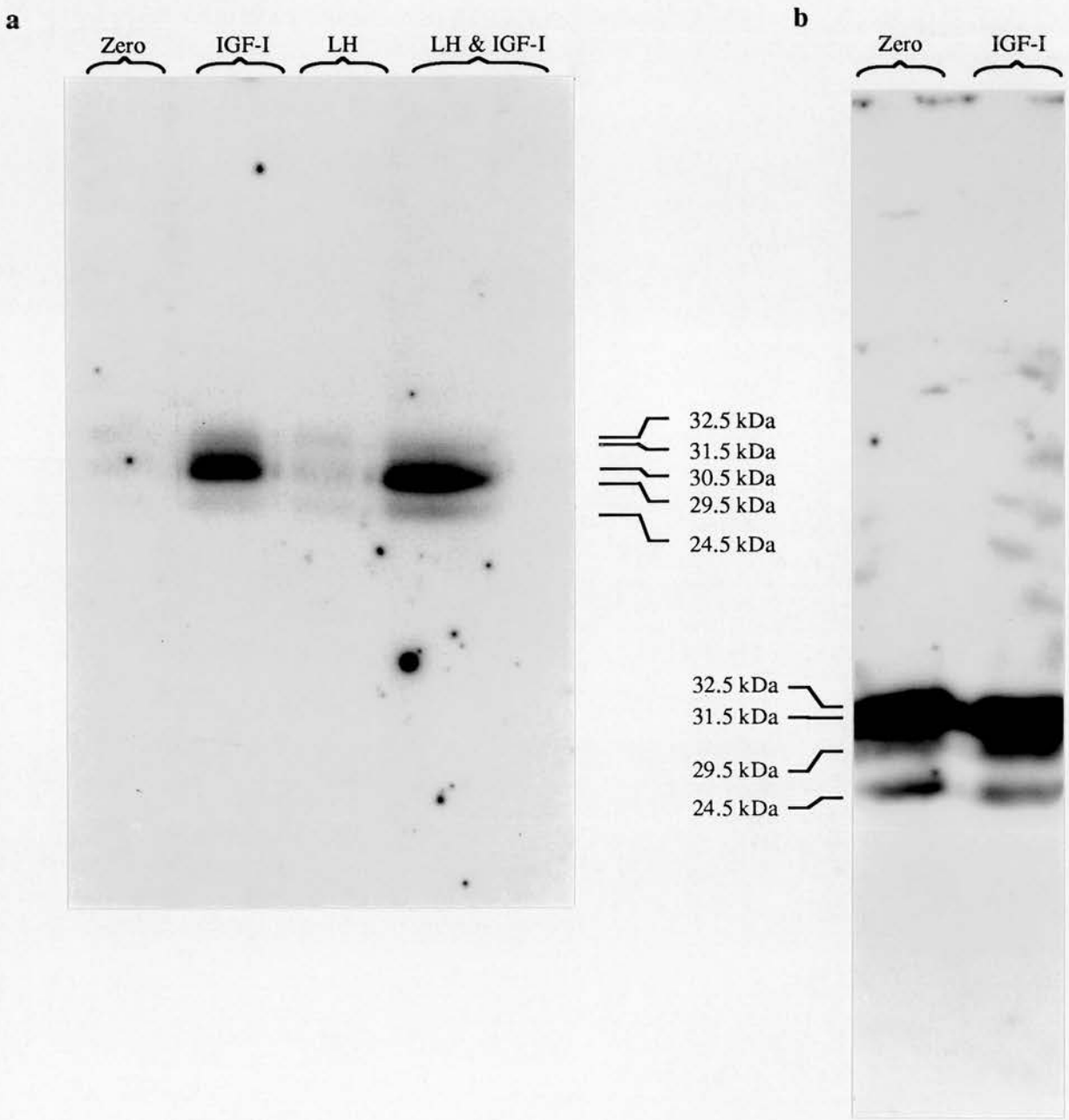

Figure 5.1 Autoradiograph of 2 Western ligand blots. Blot (a) shows the binding of [^{125}I]-IGF-I to samples of granulosa cell conditioned medium; the cells had been treated as indicated (25 ng/ml per treatment) or were untreated (zero). Blot (b) shows the binding of [^{125}I]-IGF-I to samples of thecal cell conditioned medium which were untreated (zero), or treated with IGF-I (25 ng/ml).

five binding proteins, the greatest increase being in the concentration of the 29.5kDa protein. The treatment of cells with LH alone had no effect on the production of these binding proteins by granulosa cells, and the production of binding proteins in cells treated with IGF-I was not different from that in cells treated with IGF-I and LH.

5.3. PRODUCTION OF IGFBPS BY THECAL CELLS *IN VITRO*

The endogenous production of IGF binding proteins by cultured thecal cells was compared with the production of IGF binding proteins from cells treated with IGF-I. Thecal or granulosa cells were cultured in serum free conditions or were treated with IGF-I (25 ng/ml). The thecal cell-conditioned medium (TCM) was concentrated then loaded on a PAGE gel which was blotted onto nitrocellulose. The resulting autoradiograph (Figure 5.1 b) showed that thecal cells produced four sizes of proteins with IGF-I-binding capacity (32.5, 31.5, 29.5, and 24.5 kDa). These proteins were of the same sizes as those found in GCM (Section 5.2), except for a protein of 30.5 kDa. The concentration of the proteins in untreated TCM was greater than that in untreated GCM (Figures 5.1 a, 5.1 b), however whereas treatment with IGF-I caused increased concentration of binding proteins in GCM (as seen in the previous experiments), IGF-I treatment did not appear to alter the level of expression in TCM in this experiment.

5.4. COMPARISON OF IGFBPS PRODUCED BY GRANULOSA AND THECAL CELLS WITH THOSE IN CHICKEN SERUM

IGF-I binding proteins produced by granulosa and thecal cells obtained from the ovaries of laying hens were compared with those present in the serum of the same birds. Granulosa and thecal cells were prepared as described previously, the cells were cultured and treated with IGF-I. The resulting GCM and TCM were concentrated and run on PAGE gels with a chicken serum sample. Since thecal cells were cultured at a density five times greater than granulosa cells, five times

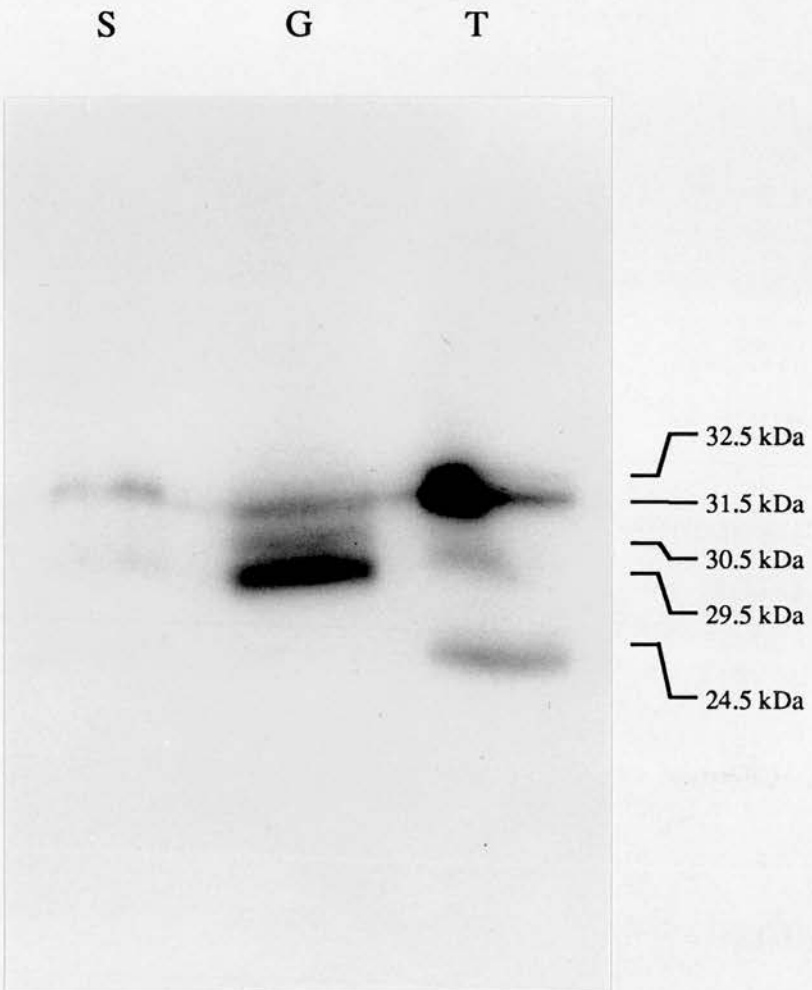

Figure 5.2 Autoradiograph of a Western ligand blot. The blot was made from a PAGE gel in which samples of serum (S), granulosa cell conditioned medium (G) and thecal cell conditioned medium (T) were run. The blot was incubated with [^{125}I]-IGF-I. The sizes of proteins to which the [^{125}I]-IGF-I bound are indicated.

more GCM was loaded than TCM so that the volume of conditioned medium per plated cell was equivalent. The gels were blotted and incubated as described in Chapter 2.

The resulting autoradiograph (Figure 5.2) showed that thecal cells treated with IGF-I produced medium containing proteins with approximate molecular weights of 32.5, 31.5, 29.5, and 24.5 kDa. GCM produced under the same conditions contained the same proteins with an additional 30.5 kDa protein. Although very faint on the autoradiograph, chicken serum contained 3 proteins with approximate molecular weights of 32.5, 29.5 and 24.5 kDa.

5.5. Binding Specificity Of [^{125}I]-IGF-I Ligand

Granulosa cells were cultured and treated with LH and IGF-I as described in Section 5.2. The concentrated conditioned medium samples were then split into two equal parts and electrophoresed in two groups of tracks on the same gel and each group was blotted separately. One of the filters was incubated with [^{125}I]-IGF-I as in the previous experiment, the other was incubated with [^{125}I]-IGF-I in the presence of 5 μg (0.5 μg/ml) of unlabelled IGF-I.

An autoradiograph of the resulting ligand blot is shown in Figure 5.3. Samples incubated without the unlabelled ligand (Figure 5.3 b) were found to contain the same binding proteins as seen in the first experiment (Figure 5.1 a). The samples incubated in the presence of unlabelled IGF-I did not bind the radiolabelled IGF-I (Figure 5.3 a). Thus, excess unlabelled IGF-I displaced the radiolabelled form from the binding sites, confirming the specificity of the IGF-I binding sites.

5.7. Summary and Discussion

Studies showing that IGF-I and IGFBP have similar distributions in human tissues (Hill *et al.*, 1989a) indicate that IGF-I binding proteins may form a complex with IGF-I on cell surfaces, and thus regulate its functions at a local

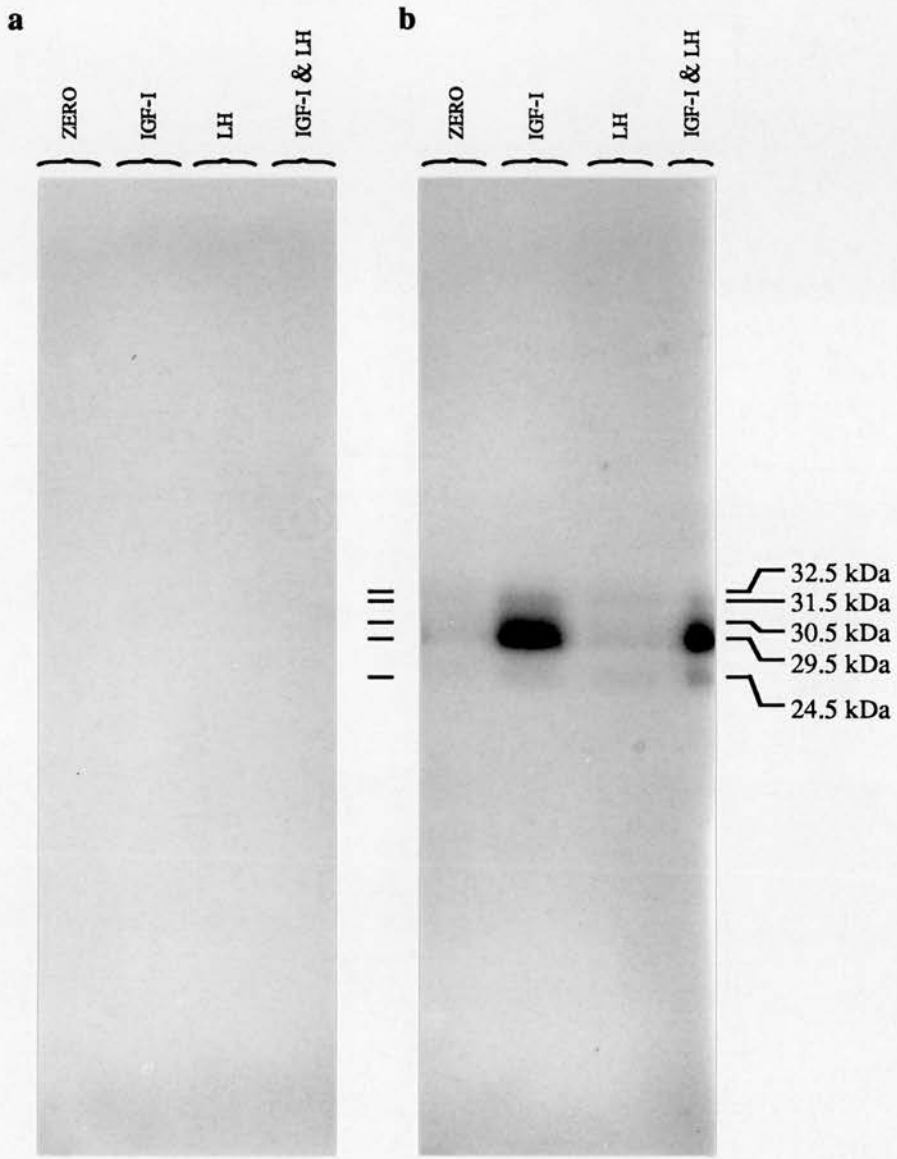

Figure 5.3 Autoradiograph of a Western ligand blot incubated with [^{125}I]-IGF-I. Samples of granulosa cell conditioned medium, from cells which had been treated as indicated (25 ng/ml per treatment) or were untreated (zero), were split and run in two sets (a) and (b). The filter (a) was incubated with unlabelled IGF-I (0.5 µg/ml).

level. The production of IGFBPs by cells and their regulation by IGF-I (Hill *et al.*, 1989b; Thrailkill *et al.*, 1990) suggests that IGFBPs may form part of an autocrine or paracrine control mechanism. Chapter 3 shows that IGF-I is produced by both granulosa and thecal cells; the experiments described in this chapter demonstrated the production of IGFBPs by these cells *in vitro* and there is thus, a potential role for IGFBPs in the regulation of IGF-I function at the local level in the chicken ovary. In order to avoid the possibility of interference from IGFBPs present in serum, the tissue culture protocol was modified so that no serum was present in the culture medium at the beginning of the treatment period.

The experiments described in Sections 5.2 to 5.4 show that granulosa and thecal cells produce 5 and 4 IGF-I binding proteins, respectively. The range of proteins is similar in size to some of those which have been identified in the rat ovary by Nakatani *et al.* These are BPs 2 and 4 with sizes of 31.3 and 25.7 kDa, respectively. Analysis of chicken serum by Armstrong *et al.* (1989) demonstrated the presence of 3 IGF binding proteins with molecular weights of 28.8, 33.2 and 40.7 kDa. The largest of these proteins could be a chicken equivalent of human IGFBP-3, which was shown by Binoux *et al.* (1991) to have similar molecular weights in human serum (41.5 and 38.5 kDa). The absence of a protein of this size from granulosa and thecal cell-conditioned medium (Sections 5.2 to 5.4) suggest that there are differences between the binding proteins present in serum and those produced by ovarian cells. This may also suggest that this putative chicken BP-3 is not involved in autocrine or paracrine control systems of IGF-I action in the ovary.

An experiment to compare proteins present in granulosa and thecal conditioned media with serum (Section 5.4) was not conclusive since the bands present in the serum tracks were faint. Although those detected were the same sizes as bands in both samples of conditioned media, it is possible that other proteins were present, but not detected because they were present in very low concentrations.

The experiments described in Sections 5.2 and 5.3 were carried out to determine whether IGFBP production by granulosa and thecal cells is regulated by IGF-I. IGFBP

production by granulosa cell cultures was stimilated by IGF-I; however, no such effect was observed for thecal cell cultures. IGF-I regulates production of IGFBPs from several cell types *in vitro*, for example IGFBP production is increased in bovine epithelial cells (McGrath *et al.*, 1991) and rabbit articular chondrocytes (Froger-Gaillard *et al.*, 1989) and reduced in human decidual cells (Thrailkill *et al.*, 1990) following treatment with IGF-I. This study shows that IGF-I also modulates the production of its binding proteins by cultured chicken granulosa cells. However, under the same conditions, no such regulation by IGF-I was observed in thecal cell cultures. This shows that cultured granulosa and thecal cells respond differently to treatment with IGF-I.

In addition to the differences in response of granulosa and thecal cells to IGF-I, the binding protein concentration in thecal cell-conditioned medium was greater, per cultured cell, than that in granulosa cell-conditioned medium. A possible explanation for these two results is that IGFBP synthesis is inhibited by a factor in granulosa cells which is down-regulated by IGF-I, and which is not present in thecal cells. Alternatively it is possible that IGF-I is stimulatory for binding protein synthesis in both granulosa and thecal cells, the stimulation threshold being lower in thecal cells. This would enable low levels of endogenously produced IGF-I to activate the production of IGFBPs in thecal cells. Endogenous IGF-I production by chicken granulosa and thecal cells *in vitro* has not been quantified, although immunocytochemical evidence presented in Chapter 3 shows that the peptide is present in both these cells in culture.

The release of IGFBPs from rat granulosa cells is modulated by FSH (Adashi *et al.*, 1990b; 1991c) and LH stimulates mitogenic activity in chicken granulosa cells, as shown by this study (Section 4.4.3) and others (Yoshimura and Tamura, 1988). Therefore, the effects of LH on IGFBP production in chicken granulosa cells were investigated, with consideration to a possible role for IGFBPs in the synergistic mitogenic actions of LH and IGF-I (Chapter 4).

The results presented in Sections 5.2 and 5.5 showed that LH did not detectably alter the production of IGFBPs by granulosa cells. In the rat, a gonadotrophin (FSH) has been shown to control IGFBP production by granulosa cells, being both stimulatory at lower doses (1-3 ng/ml) and inhibitory at higher doses (10 ng/ml) (Adashi *et al.*, 1991c); however, another gonadotrophin, LH, does not appear to regulate IGFBP production in chicken granulosa cells. This suggests that the synergistic effect of LH and IGF-I on mitogenesis in granulosa cells (Section 4.5.1.) is not due to the actions of LH on binding protein synthesis.

Thus, it has been shown that both granulosa and thecal cells release IGF binding proteins *in vitro* and that this is modulated by IGF-I but not by LH in granulosa cells. However, apart from determining their sizes, no further characterisation of these proteins was undertaken in this study. Further characterisation work, for example by immunoblotting with antisera raised against mammalian IGFBPs, could indicate similarities or differences of these proteins with the mammalian IGFBPs.

CHAPTER 6: IGF RECEPTORS IN THE AVIAN OVARY

6.1. INTRODUCTION

The experiments were conducted to determine whether the cells used in the *in vitro* studies described in Chapters 4 and 5 have receptors for insulin-like growth factors. The cells used in this study were granulosa and thecal cells mixed from F1, F3 and F5 follicles of laying hens.

In the experiments described below, the IGF-I receptors were measured by competitive ligand binding studies. These compared the relative affinities of the ligands IGF-I, IGF-II and insulin for IGF-I binding sites on the basis of their competition with radiolabelled IGF-I.

6.2. DEMONSTRATION OF IGF RECEPTORS IN GRANULOSA AND THECAL CELLS

Granulosa and thecal cells were cultured in 48-well culture plates and then treated with [^{125}I]-IGF-I (20,000 cpm/well) alone or with unlabelled IGF-I, IGF-II or insulin. Ligands were added separately in a range of doses and each treatment was repeated three times (Figure 6.1). As a control for non-specific binding of the label to the plates, wells without cultured cells were incubated with [^{125}I]-IGF-I with and without unlabelled IGF-I.

Radiolabelled [^{125}I]-IGF-I bound to the granulosa and thecal cells with an efficiency which ranged from 0.9 to 1.5% of the total counts added per well. Non-specific binding of label to plates in which no cells were present was less than 0.1% of the total added per well.

The binding of [^{125}I]-IGF-I to both granulosa (Figure 6.1 a) and thecal cells (Figure 6.1 b) was displaced in a dose-dependent manner by the addition of increasing concentrations of unlabelled IGF-I, IGF-II or insulin (Figure 6.1). Values of IC_{50} for granulosa cells were 2.87 ± 0.27 (s.e.m.) pmol/l, 3.31 ± 0.97 pmol/l and 248.2 ± 39.4 pmol/l for hIGF-I, hIGF-II and insulin respectively; for thecal cells the values were

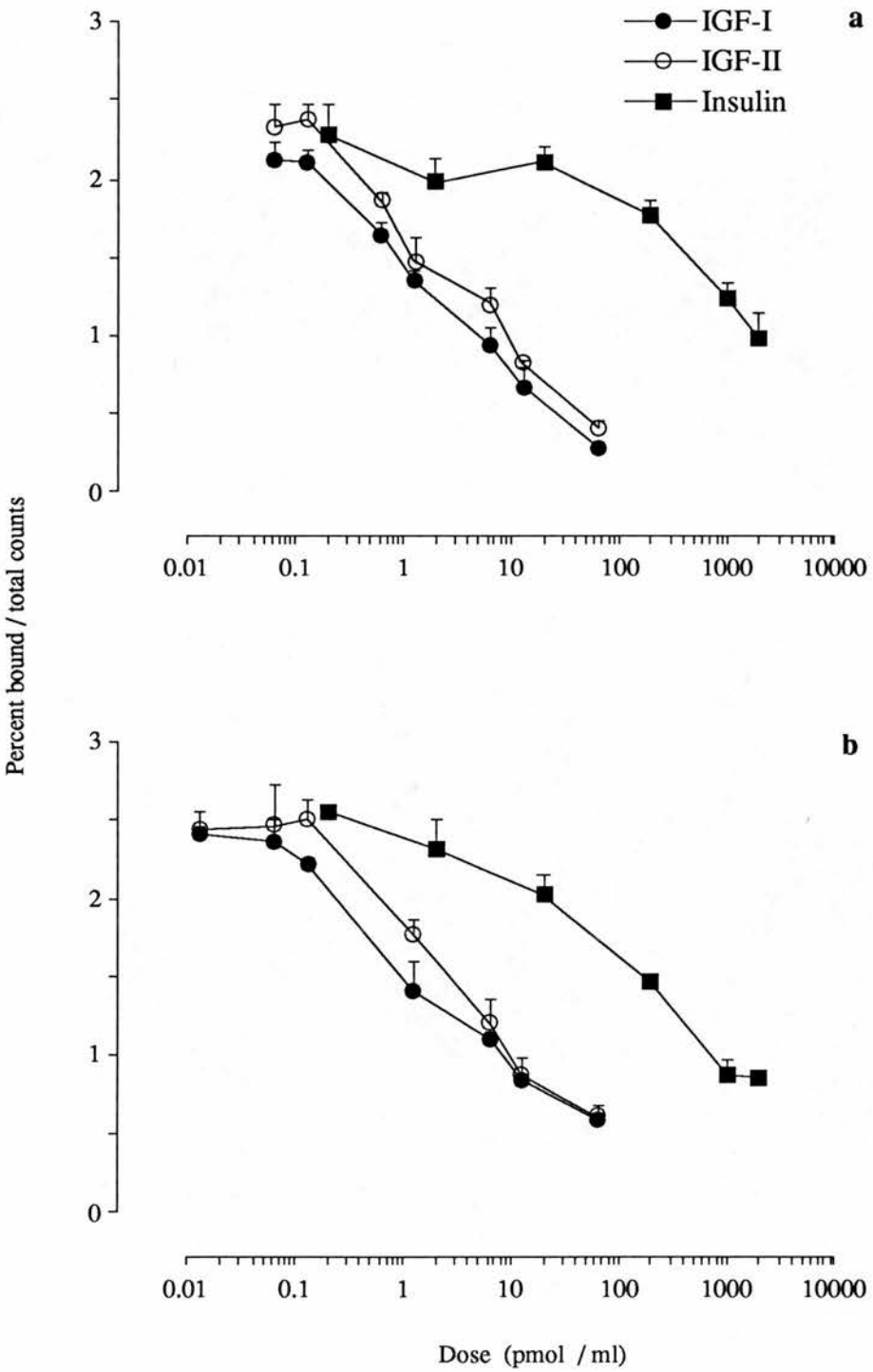

Figure 6.1. Binding of [^{125}I]-IGF-I to (a) granulosa cells and (b) thecal cells in the presence of increasing concentrations of unlabelled IGF-I, IGF-II or insulin. Cells were cultured and the binding studies performed as described in the text. Values are the means of triplicate cultures, standard deviations are shown as vertical bars.

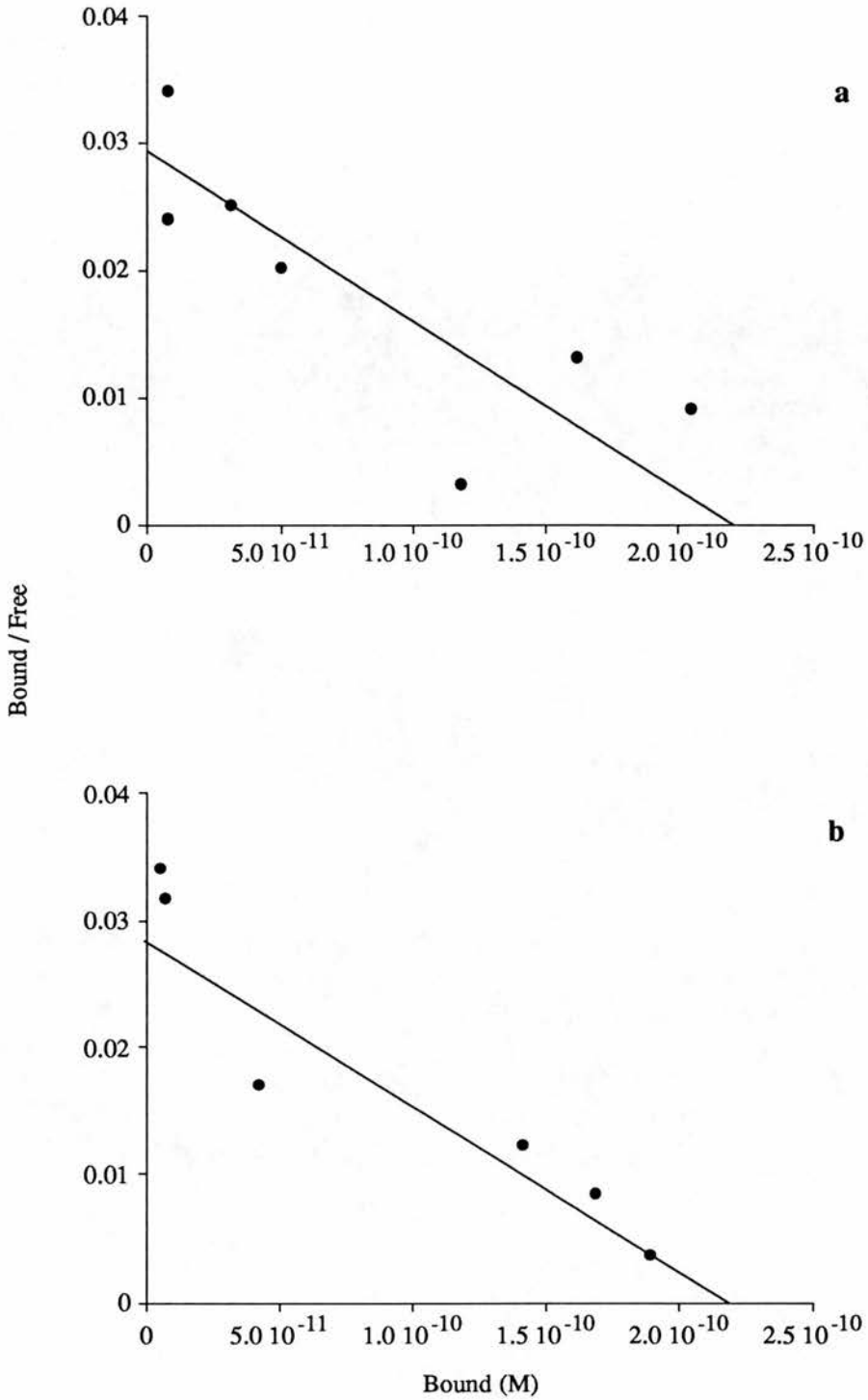

Figure 6.2. Scatchard analysis of the data from the experiments described in figure 6.1. a) analysis of [125-I] IGF-I binding to granulosa cells. b) analysis of [125-I] IGF-I binding to thecal cells. The analysis was performed with the LIGAND analysis program.

1.36 ± 0.15 (s.e.m.) pmol/l, 4.7 ± 0.74 pmol/l and 249.3 ± 31.9 pmol/l for the same ligands; these values were calculated with the ALLFIT program (De Lean *et al.*, 1978). Thus the order of potency with which the unlabelled peptides displaced [^{125}I]-IGF-I from both granulosa and thecal cells was IGF-I > IGF-II >Insulin.

The affinity constant (K_a) and binding capacity (B_{max}) of IGF-I for the binding site on granulosa cells were 7.36 ± 17.3 litres/nmol and 215 ± 40.1 fmol/well respectively; those on thecal cells were 7.7 ± 27.2 litres/nmol and 214 ± 90 fmol/well respectively (300 ng DNA per well), these were calculated using the LIGAND statistical program (Munson and Rodbard, 1980). The analysis shows that the characteristics of the IGF-I binding sites on granulosa and thecal cells are the same (Figure 6.2). Comparison of one and two binding-site models for the Scatchard analysis indicated that the one binding site model was most appropriate for the data for both the granulosa and thecal cell studies. The experiments were confirmed, once for each cell type.

6.3. Regulation Of Granulosa Cell IGF Receptors By LH

Granulosa cells were prepared and cultured as described above. After the restriction period of culture, cells were treated with 50 ng/ml oLH in medium M199 with 0.1% FCS or left untreated in medium. A treatment period of 6 hours was followed by incubation with labelled and unlabelled peptides as described above.

Treatment of granulosa cells with LH appears to have no appreciable effect on the order of affinity of IGF-I, -II and insulin for IGF-I binding sites (Figure 6.3). Scatchard analysis of the data produced K_a and B_{max} values for IGF-I of 7.51l/nmol, 220 fmol/well and 9.98 l/nmol, 383 fmol/well for the control and LH treated cells, respectively. These estimates are essentially the same as observed for cells treated with IGF-I and suggest that treatment of granulosa cells with LH does not affect the affinity of IGF-I for its receptor or the number of IGF receptors on granulosa cells. However, there was insufficient data for analysis using LIGAND, therefore the values shown

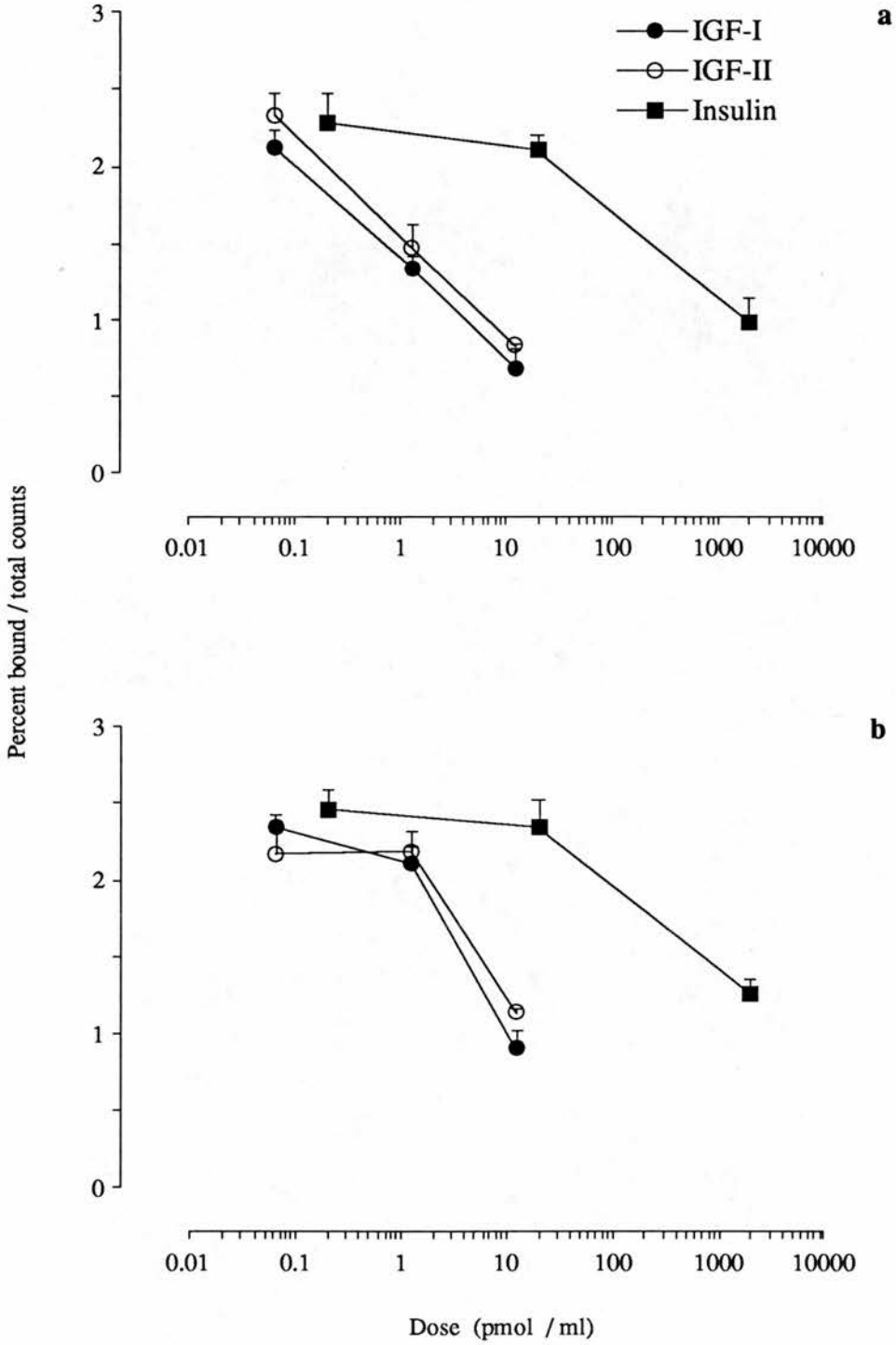

Figure 6.3. Binding of [^{125}I]-IGF-I to granulosa cells in the presence of increasing concentrations of unlabelled IGF-I, IGF-II or insulin. Cells were cultured a) untreated or b) treated with 50 ng/ml LH. Values are the means of triplicate cultures, standard deviations are shown as vertical bars.

were estimates produced from an eqilibrium binding data analysis (EBDA) program which is not as accurate as LIGAND.

6.4. Summary And Discussion

Cultured granulosa and thecal cells were shown to have binding sites for IGF-I and that IGF-I, IGF-II and insulin displace labelled IGF-I from these sites with relative potencies similar to those described by Rechler *et al.* (1980) for human fibroblasts and BRL 3A2 cells. This indicates that the binding sites for IGF-I on chicken granulosa and thecal cells are type I IGF receptors. The data were consistent with a one binding site model, which supports other work showing that there is only one IGF receptor type in chicken cells (Bassas *et al.*, 1988; Canfield and Kornfeld, 1989; Duclos and Goddard, 1990). In this respect, chicken granulosa cells appear to differ from rat granulosa cells which have been shown to posses two IGF receptor types (Davoren *et al.*, 1986). There were no significant differences of receptor affinities or binding capacities for IGF-I between granulosa and thecal cells. Thus there is no evidence to suggest that the differences observed in the growth response of these cells, as described in Sections 4.4.1. and 4.4.3., are due to differences in their IGF receptors.

The experiment described in Section 6.3 explored the possible regulatory effects of LH treatment on the IGF receptors of granulosa cells and showed that LH had no effect on either receptor number or affinity. Thus, the synergistic effects of IGF-I and LH on [3H]-thymidine uptake (Chapter 4) does not appear to be modulated through an effect on the IGF receptor. However, the data did not support detailed analysis due to the small scale of the experiment (which was not repeated). Thus no great significance should be attached to these findings

The experiments in this chapter showed that insulin displaced IGF-I from its receptors in chicken granulosa cells and therefore possibly act through them. However, its potency was much lower than IGF-I or IGF-II suggesting that *in vivo*, the IGF receptor mediates the effects of IGF-I and IGF-II but not insulin. Insulin is more likely to mediate its effects on granulosa cells via its own receptor. In support of this view,

Webb and McBride (1991) working with mammalian (ovine) granulosa cells showed that insulin and IGF-I have dose-dependent effects on [^{3}H]-thymidine incorporation by these cells with ED_{50}s of 6.8 and 3.8 nM respectively, supporting the hypothesis that these peptides act through separate receptors.

In conclusion, chicken granulosa and thecal cells posses IGF receptors with characteristics similar to those described for type I IGF receptors in previous studies, and that there are no differences in these characteristics between granulosa and thecal cells *in vitro*.

CHAPTER 7: GENERAL DISCUSSION

It is well established that IGF-I has both mitogenic and steroidogenic properties in the mammalian ovary (see Chapter 1 for references). This study investigated the nature and some of the actions of IGF-I in the ovary of the domestic hen. It was established that the IGF-I gene is expressed in the granulosa and thecal cells of the four largest pre-ovulatory follicles and that IGF-I is present in both these tissues *in vivo* and *in vitro*. Secondly it was shown that IGF-I stimulates DNA synthesis in a dose-dependent manner in granulosa and thecal cells *in vitro*; and that the gonadotrophin, LH has a similar effect on granulosa cells but not thecal cells. Further, LH and IGF-I act synergistically to stimulate DNA synthesis in granulosa cells. Thirdly, it was found that granulosa and thecal cells produce IGF-I binding proteins *in vitro*, which in granulosa cells are regulated by IGF-I. Finally, it was demonstrated that IGF-I binding sites are present on granulosa and thecal cells *in vitro* and that these are characteristic of IGF type-I receptors.

In the original somatomedin hypothesis, IGF-I (somatomedin-C) was considered to be a growth hormone-dependent factor which was synthesised primarily in the liver and then circulated around the body where it mediated the actions of growth hormone on skeletal growth (Van Wyk *et al.*, 1974; Van Wyk, 1984; Froesch *et al.*, 1985). However, since this hypothesis was proposed, it has been established that IGF-I is synthesised in many diverse extra-hepatic tissues. This indicates that it also has an autocrine or paracrine role.

The known interactions of IGF-I in chicken thecal and granulosa cells with respect to mitogenesis are shown in the summary diagram in Figure 7.1. Most of the information represented was elucidated by the studies described in this Thesis.

This work has shown that the IGF-I gene is expressed in chicken ovarian cells, that the peptide itself and IGF receptors are present in the same cells and that IGF-I affects the growth of these cells. This indicates that IGF-I is synthesised locally in the chicken ovary and that it may act in a paracrine/autocrine manner. Since both granulosa

and thecal cells were shown to produce IGF-I and posses IGF-I receptors, it seems that the growth factor has autocrine/paracrine actions in either or both cell types.

A system of local control of IGF-I is implicit in an autocrine/paracrine model. One of the factors which has been found to regulate the effects of IGFs locally are the IGFBPs. The pattern of IGFBP occurrence in human tissues is similar to that of the IGF peptides (Hill *et al.*, 1989(b)), This led to suggestions by these and other workers that IGFs and IGFBPs are mutually regulated. More recently, several studies have shown that administration of IGFs increases IGFBP production from cells both *in vitro* and *in vivo* (e.g. Zapf *et al.*, 1989; Froger-Gaillard *et al.*, 1989). When this information is coupled with earlier work which showed that IGFBPs inhibit the effects of IGF-I on cells (Knauer and Smith, 1980; Blat *et al.*, 1988), the basis for a local mechanism regulating IGF-I actions by IGFBPs emerges. Simply, this would involve cells responding to increased IGF-I concentrations by increasing IGFBP synthesis, which then attenuate the effects of the peptide by forming complexes with it (Figure 7.1). Modification of IGFBPs by proteases may inhibit the formation of these complexes (Hossenlopp *et al.*, 1990; Schmid *et al.*, 1991) and thus regulate the availibility of free IGF-I to cells, introducing a further level of control on IGF-I activity.

The work presented in this Thesis shows that IGFBP production by chicken granulosa cells is stimulated by the administration of IGF-I and is consistent with the mechanism described above. As further evidence of this mechanism operating in the ovary, IGFBPs are produced by mammalian ovarian cells (Suikkari *et. al.*, 1989; Nakatani *et al.*, 1991; Erickson *et al.*, 1992) and have been shown to inhibit the stimulatory effects of IGF-I on FSH-induced oestradiol synthesis by rat granulosa cells (Shimasaki *et al.*, 1990(a)). Also, IGF-I was recently shown to stimulate the production of 2 IGFBPs by porcine granulosa cells in a dose-dependent manner (Grimes and Hammond, 1992). A stimulatory role of IGFBPs in the ovary can not be ruled out since they increase cell proliferation in porcine muscle cells, human and chick fibroblasts and baby hamster kidney cells (Clemmons *et al.*, 1986; Elgin *et al.*, 1987;

Blum *et al.*, 1989). However, the other reports of IGFBP actions in the ovary have shown them to be inhibitory (Bicsak et al., 1990; 1991). Further evidence of the importance of IGFBPs in regulating ovarian follicular development was provided by Nakatani et al. (1991), who showed that the IGFBP-4 gene is expressed exclusively in the granulosa cells of atretic follicles in rats. Thus evidence is shown in this Thesis which is consistent with the ovarian actions of IGF-I being regulated by IGFBPs by means of a negative feedback loop in the domestic hen (see Figure 7.1); this is supported by similar work in mammalian species.

The gonadotrophin, LH, was shown in these studies to be mitogenic for granulosa cells and therefore acts in a similar manner to IGF-I. When LH and IGF-I were added together to cultured granulosa cells, there was a synergistic effect on DNA synthesis. Further experiments are necessary in order to determine the nature of the synergistic relationship between IGF-I and LH with respect to cell growth. One possible area of investigation suggested by other studies is the role of cAMP. The steroidogenic effects of FSH on rat granulosa cells are mediated by cAMP, which is also involved in mitogenic actions of both FSH and LH on chicken granulosa cells (Yoshimura and Tamura 1988; 1991; Adashi *et al.*, 1991b). Additionally, treatment of rat granulosa cells with IGF-I and FSH results in an increase in accumulation of cAMP in conditioned media which is greater than that seen after treatment with FSH alone (Adashi *et al.*, 1986b). Thus, it is possible that cAMP is involved in the synergistic effects of IGF-I and LH shown here.

In the light of evidence showing that another gonadotrophin, FSH, regulates IGFBP release from rat granulosa cells (Adashi *et al.*, 1991c), it seems possible that LH could exert its mitogenic effects on granulosa cells by reducing IGFBP production and thus reducing the inhibitoty effects on IGF-I due to these binding proteins. The possible regulatory effects of LH on IGFBP release from chicken granulosa cells were therefore investigated. However, the results showed no such effects, suggesting that LH does not regulate mitogenic activity of IGF-I via an IGFBP system. A further experiment to examine the effects of LH on granulosa IGF receptor number and

affinity, showed that there was no effect. Thus LH does not appear to regulate the mitogenic actions of IGF-I via the IGF receptor.

IGF-I, is likely to be one of several growth factors involved in the growth of follicles, two more are likely to be epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) which together with IGF-I have been identified as being important to the control of the cell cycle (Pledger *et al.*, 1978; Leof *et al.*, 1982). Recent reports have shown that EGF has steroidogenic and mitogenic effects on the cells of chicken pre-ovulatory follicles (Onagbesan *et al.*, 1990; Peddie *et al.*, 1991; Peddie, 1992). EGF also acts synergistically with IGF-I to induce mitogenesis in porcine granulosa cell (May *et al.*, 1988). Other factors, most notably gonadotrophins have been shown to act synergistically with IGF-I to stimulate steroidogenesis in granulosa cells (e.g. Adashi *et al.*, 1985b; Veldhuis *et al.*, 1987). In this study, a synergistic effect of LH and IGF-I on mitogenesis was observed and the observation that IGF-I and low concentrations of serum are mutually synergistic in the same way, suggests that serum contains additional factors which act synergistically with IGF-I.

The synthesis of IGF-I is known to be influenced by many factors, depending on tissue or cell type. For example, IGF-I production by porcine granulosa cells is stimulated by LH, FSH, GH, oestradiol, EGF and TGF-α (Hsu and Hammond, 1987; Mondschein and Hammond, 1988). Thus it is probable that in an autocrine/paracrine system as described above IGF-I activity would be regulated by proximal factors such as the pituitary hormones. One such hormone, GH, has been shown to increase IGF-I gene expression in cultured rat hepatocytes (Johnson et al., 1989) and to elevate IGF-I levels in rat ovarian tissues (Davoren and Hsueh, 1986). A recent report showed that GH stimulates the development of ovarian follicles in the domestic hen, and that it is associated with an increase in plasma IGF-I concentrations (Williams *et al.*, 1992). Thus GH may be a candidate for proximal regulation of IGF-I action in the avian ovary.

The studies of IGF-I stimulation of IGFBP synthesis and LH stimulation of DNA synthesis showed that granulosa and thecal cells behave differently. The

granulosa cells were responsive to both treatments, but no effects were observed in thecal cells. The conclusions drawn from this is that the granulosa layer of cells is more sensitive to the effects of IGF-I (both alone or combined with LH) than the adjacent thecal layer in the follicles of domestic hens. Since the IGF-I gene was shown to be expressed in both tissues, it is possible that IGF-I synthesised in thecal cells could exert effects on the adjacent granulosa cells and thus act in a paracrine manner. However, further studies involving co-culture of both cell types are required before further conclusions can be drawn.

This study clearly shows that IGF-I affects the growth of avian granulosa and thecal cells. The involvement of IGF-I with ovarian growth and development was originally indicated by studies showing that IGFs are present in mammalian follicular fluid *in vivo* (Hammond *et al.*, 1982). This was reinforced by studies showing production by cells *in vitro* and further showing that IGF-I has mitogenic and steroidogenic roles in mammalian granulosa cells (Baranao and Hammond, 1984; Adashi *et al.*, 1985b). Thus IGF-I is involved in the development of the avian pre-ovulatory ovarian follicle. The nature of the involvement of IGF-I in the ovary of the domestic hen appears to be complex, with an autocrine/paracrine IGF-I system complete with ligands, receptors and binding proteins which interact with at one factor (LH) and possibly others. The role of IGF-I in the ovary of the domestic hen, as elucidated in this Thesis and by other studies, is summarised in Figure 7.1.

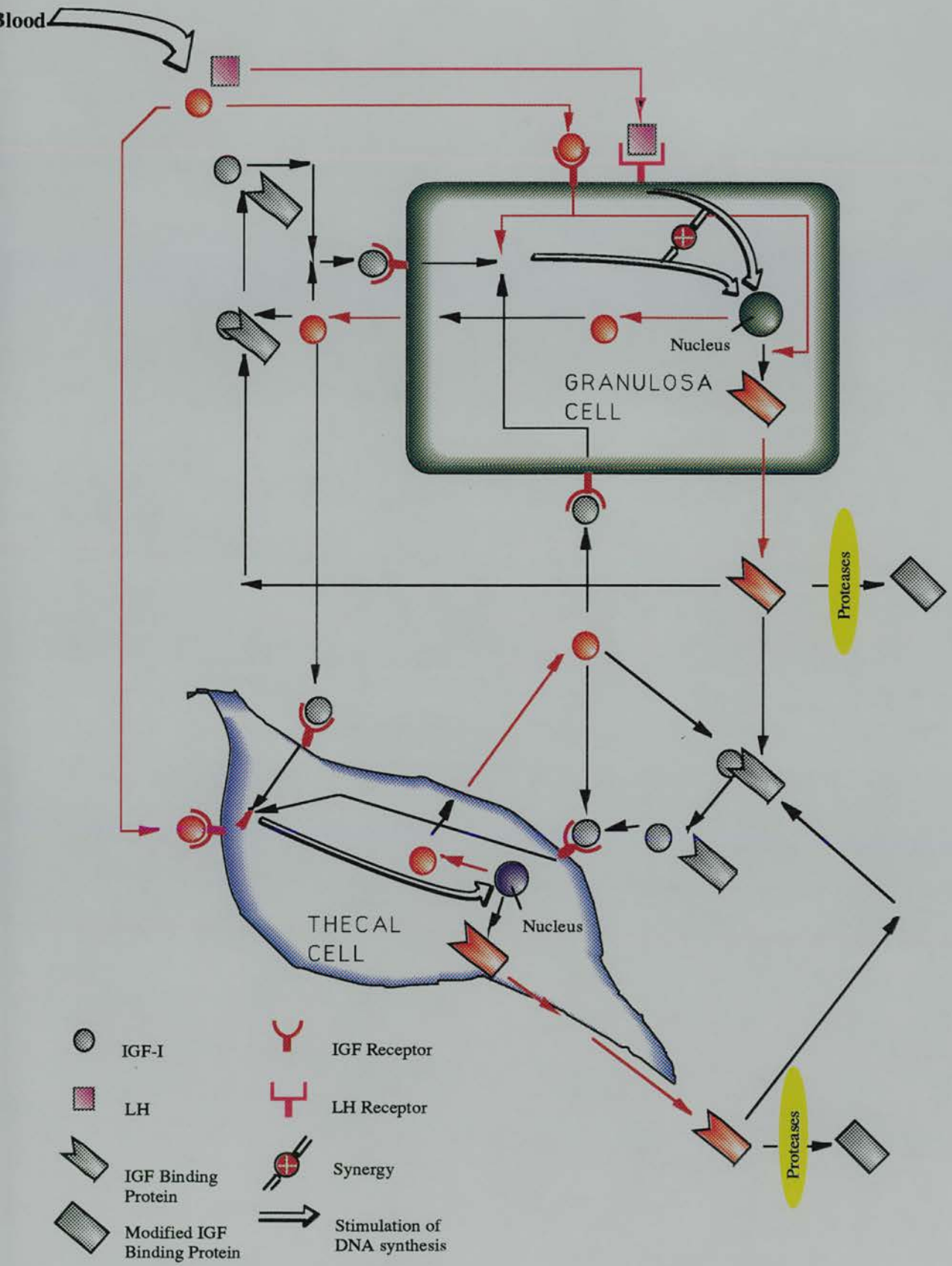

Figure 7.1 Summary of the interactions of IGF-I in granulosa and thecal cells of the domestic hen. Elements in red are described in this thesis, elements in grey are speculation based on mammalian studies. Elements in pink were elucidated by other studies.

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Addendum

Davoren, J. B. and Hsueh, A. J. W. (1984). Insulin enhances FSH-stimulated steroidogenesis by cultured rat granulosa cells. Mol. Cell Endocrinol. 35, 97-105.